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CENTRE D'ECOLOGIE ET DE TOXICOLOGIE
DE L'INDUSTRIE CHIMIQUE EUROPEENNE

EUROPEAN CHEMICAL INDUSTRY ECOLOGY
AND TOXICOLOGY CENTRE

Avenue Louise 250, Bte 63
B - 1050 Brussels
Telex APME B 61 523
Téléphone : (02) 649 94 80
T.V.A. 418.344.469

ECETOC Statement · - June 1987: Current Results from European Chemical Industry-Sponsored Research into Species Differences in the Toxicology of Methylene Chloride

Following the 1986 submission to Regulatory Agencies of seven reports describing the results of the CEFIC-sponsored research into the species differences in methylene chloride carcinogenicity, several areas for further research were identified. These included (1) an improved methodology for the detection of the glutathione-S-transferase pathway in human and animal tissues, (ii) the analysis of further human tissue samples for the presence of this pathway, (iii) an investigation of the enzyme changes associated with the sub-acute lesion seen in the mouse lung Clara cell and (iv) identification of the source of methylene chloride derived CO_2 in order to evaluate the relative utilisation of the glutathione-S-transferase and cytochrome P-450 pathways at different dose levels.

This research was undertaken to facilitate more accurate extrapolation both across species and from high to low dose levels and to better define the role of the Clara cell in the development of lung tumours in methylene chloride exposed mice. An outline of the proposed studies was given in a submission made to the US agencies in February 1987 and a brief statement dated May 6th 1987 informed the agencies that glutathione-S-transferase metabolism of methylene chloride had been detected in hamster and human liver tissue, in addition to that previously reported in rat and mouse liver. This present statement summarises the results of the current research programme. Full reports of these studies will be available in October 1987.

I. Glutathione-S-transferase metabolism of methylene chloride.

Methylene chloride is metabolised by a glutathione-S-transferase isoenzyme to formaldehyde. Therefore comparisons of the relative activity of this enzyme in different species should be based on the formation of this product. However, assay of formaldehyde is difficult, firstly because it may be further metabolised by other enzymes present in the liver fractions and secondly because its detection is based on a colorimetric assay of limited sensitivity.

In the previous studies (Green et al 1987) the loss of formaldehyde by further metabolism was minimised by careful selection of the cofactors used. Glutathione and NAD^+ are both required for the further metabolism of formaldehyde, but glutathione alone is required for the metabolism of methylene chloride to formaldehyde. Glutathione is not consumed during the metabolism of methylene chloride and therefore high concentrations of this cofactor are not required. Thus at the endogenous level of glutathione and in the absence of added NAD^+ the formation of formaldehyde was maximised and its further metabolism was minimised. The conditions do not necessarily give the maximum rate of metabolism of methylene chloride, but they do give a measurable rate without significant loss of the end product. Subsequent studies with radioisotopes under conditions of optimal methylene chloride metabolism have shown that the conditions used for the formaldehyde study do not affect the relative species differences in the rate of methylene chloride metabolism by the glutathione-S-transferase pathway.

Using the formaldehyde assay, methylene chloride related glutathione-S-transferase activity could not be detected in hamster and human liver tissues. Alternative methods were therefore developed to assay these tissues for the glutathione-S-transferase isoenzyme responsible for the metabolism of methylene chloride. These more sensitive methods based on radioisotopes have confirmed the species differences measured with the formaldehyde assay and have been able to detect low levels of activity in both hamster and human liver tissues.

Two methods have been used based on either the release of Cl-36 ion from Cl-36 labelled methylene chloride or on the coversion of C-14 labelled methylene chloride to water soluble products. The use of the C-14 technique was necessary because of the limited availability of the Cl-36 labelled material. Both radiochemical techniques have a weakness in that they do not measure a specific

product and consequently the result may be confounded by the presence of radiochemical impurities in the test compounds. This effect is greater at low rates of metabolism, such as in hamster or human tissue, than at the high rates found in mouse tissue. At low rates of methylene chloride metabolism interference from radiochemical impurities will result in over-estimation of the methylene chloride dependant rate. For example radiochemicals are typically supplied with a purity of 98-99%. Using a 35mM concentration of methylene chloride mouse tissues are able to convert 10-15% of the substrate to product, whereas hamster and human tissues convert less than 1% and may therefore be significantly influenced by the conversion of impurities, present at concentrations up to 1%, to water soluble or Cl-36 ionic products.

Addition of varying concentrations of glutathione to the radioisotope assays demonstrated an optimum concentration of 5-10mM. Glutathione was not added to the formaldehyde assay for the reasons given above. As a consequence of the different levels of this co-factor the rate measured with the radioisotopes were approximately double those found with the formaldehyde assay. However the species differences were identical. Using a 35mM concentration of C-14 methylene chloride the rates were 26.3, 5.4 and 1.7nmol methylene chloride/ min/mg protein for mouse, rat and hamster liver cytosol respectively. The rates for the mouse and rat using the formaldehyde assay were 11.2 and 2.1nmol formaldehyde/min/mg protein respectively (CTL/R/879). Human liver cytosol samples (n=7) gave a range of rates from 0.04 to 0.96nmol/min/mg protein with a mean value of 0.44nmol/min/mg when assayed with Cl-36 methylene chloride (35mM). Using the same isotope, the rate for hamster liver cytosol was 1.2nmol/min/mg.

In summary, the relative rates of metabolism of methylene chloride by the glutathione-S-transferase pathway, measured with radioisotopes were: mouse 26.3, rat 5.4, hamster 1.2-1.7, human 0.04-0.96nmol/min/mg protein at a substrate concentration of 35mM.

II. Analysis of further human tissues.

Ten human liver samples have now been assayed, three with the formaldehyde assay, six with the Cl-36 techniques and one by both methods. The results of the assay with these samples are given in the previous section of this statement. At the present time human lung tissue is not available for these

studies. In view of the low activity in human liver it seems highly unlikely that activity would be detected in human lung.

III. Mouse lung Clara cell studies.

The lesion seen in the mouse lung Clara cell after a single exposure to methylene chloride could significantly influence the metabolism of methylene chloride in that cell type. The lesion is confined to the smooth endoplasmic reticulum in the Clara cell and similar effects with other chemicals have resulted in a marked loss of cytochrome P450. It was postulated that the soluble cytosolic glutathione-S-transferase would not be affected by this lesion resulting in preferential metabolism of methylene chloride by this pathway in Clara cells with the lesion. Our previous studies have suggested that glutathione-S-transferase metabolism of methylene chloride is responsible for the species difference in carcinogenicity. Thus the loss of the detoxifying cytochrome P450 pathway with no effect on the glutathione pathway could significantly increase the risk to that cell type. Our present studies have been able to confirm that methylene chloride treatment selectively reduces cytochrome P450 metabolism. Following a single 6hr exposure to 4000ppm methylene chloride the ability of whole lung microsomal fractions to metabolise methylene chloride to carbon monoxide is reduced by almost half whereas the activity of the cytosolic glutathione-S-transferases is unaffected. The use of monoclonal antibodies to cytochrome P450 isoenzymes demonstrated that the loss of cytochrome P450 occurred almost exclusively in the Clara cell. Further studies are in progress to investigate the effects of prolonged exposure to methylene chloride on Clara cell metabolising enzymes.

IV. The relationship between dose and route of metabolism.

The Michaelis enzyme affinity constants (Km) for the two pathways (Green et al 1987) suggest that the cytochrome P450 pathway is the major pathway at low dose levels and the glutathione pathway is active mainly at high dose levels when the cytochrome P450 pathway has saturated. The relative utilisation of the two pathways of methylene chloride metabolism in vivo has for many years, been based on the assumption that the activity of the cytochrome P450 pathway could be determined by measuring exhaled carbon monoxide and that the glutathione pathway could be quantitatively assessed by measurement of exhaled carbon dioxide.

Recent studies using metabolic inhibitors (Gargas et al 1986, Reitz et al 1986) suggest that CO_2 is also derived from the cytochrome P450 pathway, hence conclusions based on the relative molar amounts of CO and CO_2 could be in error. Clearly accurate assessment of these pathways in vivo is crucial for extrapolation from the high exposure levels used in the NTP studies to the low levels found during human occupational exposure. Such an assessment is dependant upon identifying the source of CO_2 at different dose levels.

This has now been achieved by using radiolabelled stable isotopes of methylene chloride. CO or CO_2 derived from the cytochrome P450 pathway will show a stable isotope effect, i.e. a reduction in the rate of formation, on changing from CH_2Cl_2 to CD_2Cl_2 whereas CO_2 derived from the glutathione pathway will not.

Using this principle it has been shown unequivocally that CO_2 is derived from both metabolic pathways. This was demonstrated by measuring exhaled C-14 carbon monoxide and C-14 carbon dioxide during 6hr exposures of mice to both C-14 CH_2Cl_2 and C-14 CD_2Cl_2 at concentrations of 100, 500 and 4000ppm. At 100ppm 71% of the exhaled CO_2, equivalent to 121µmol CO_2/kg body weight/hr, is derived from the cytochrome P450 pathway. At 500ppm 55% (135µmol/kg/hr) is derived from cytochrome P450 metabolism, the remainder coming from the glutathione pathway. At this dose level the cytochrome P450 pathway saturates and hence CO_2 from this pathway does not exceed 135µmol/kg/hr. At higher dose levels the increasing levels of CO_2 arise from increasing utilisation of the glutathione pathway so that at 4000ppm the majority (78%) of the 528µmol CO_2/kg/hr is from this pathway. In summary, at low doses of 100ppm the major source of exhaled CO_2 is cytochrome P450 metabolism, at high doses the converse is true, glutathione metabolism providing most of the exhaled CO_2.

This analysis of the source of CO_2 in conjunction with the measurements of the rate of formation of CO at the different dose levels, provides a complete assessment of the utilisation of the two pathways at different dose levels. At 100ppm, 200µmol/kg/hr of methylene chloride are metabolised by the cytochrome P450 pathway and 47µmol/kg/hr by the glutathione pathway. At 500ppm the figures are 275 and 112µmol/kg/hr, and at 4000ppm 288 and 414µmol/kg/hr for the cytochrome P450 and glutathione pathways respectively in each case.

Studies in rats using one of these isotopes, C-14 CH_2Cl_2, have shown CO_2 to be a relatively minor metabolite in this species in comparison to mice. The total rate of formation from both pathways at 500ppm was 33µmol/kg/hr increasing to only 55µmol/kg/hr at the 4000ppm dose level. These studies emphasise the species difference in the utilisation of the glutathione pathway at high doses in rats and mice. At 4000ppm exposures rats produce 55µmol/kg/hr, mice 528µmol/kg/hr.

Conclusions

Low rates of glutathione-S-transferase metabolism of methylene chloride have been detected in human liver tissue. These levels are significantly below those in hamster and rats, two species where lung and liver cancer was not seen even after a life-time exposure to 3500/4000ppm methylene chloride. Markedly higher rates of metabolism by this pathway in vitro were found in mice than in any other species. Significantly the mouse/rat difference measured in vitro was quantitatively similar to that measured in vivo suggesting that in vitro studies are an accurate reflection of the in vivo utilisation of this pathway. Hence it is reasonable to assume that the in vitro hamster and human results reflect the in vivo metabolism of methylene chloride in these species.

Stable isotope studies have shown that over 70% of the CO_2 exhaled by mice exposed to 100ppm methylene chloride is derived from the cytochrome P450 pathway. Comparisons of the rate of CO_2 production at 4000ppm exposures have demonstrated the marked species difference between rats and mice which is the basis of species difference in carcinogenicity.

The sub-acute lesion in the mouse lung Clara cell has been shown to result in decreased cytochrome P450 metabolism without a similar effect on the glutathione-S-transferase pathway. As a result these cells will be at increased risk at high exposures. At the same time cellular damage and regeneration may potentiate the development of mouse lung tumours.

Implications for risk assessment.

It is now clear that the complex metabolism of methylene chloride involving two pathways with different enzyme/substrate affinities and saturation levels cannot be adequately described by linear extrapolation from high to low dose. This can only be achieved by taking into account the metabolic and kinetic constants that describe the behaviour of methylene chloride at different doses in each species.

Equally the marked species difference in the utilisation of the glutathione pathway cannot be described by cross species extrapolations based on body weight or surface area corrections. Accurate risk assessment should therefore be based on the internal dose of metabolites from the glutathione-S-transferase pathway utilising species dependant physiological and pharmacokinetic parameters to quantify the internal dose. As a result of this research programme it is now possible to undertake this type of risk assessment using the mathematical model developed by Anderson et al (1987). The glutathione pathway has clearly been identified as the pathway of risk. In vivo pharmacokinetic data is now available at up to five dose levels from 100 to 4000ppm, including blood levels of the parent chemical, post exposure elimination of parent chemical and the elimination of CO and CO_2 both during and post exposure and studies to identify the sourse of CO_2 at different dose levels. Furthermore this data is available from studies using the same species, strains and exposure conditions used in the NTP cancer bioassay. It is now possible to determine from this data the in vivo metabolic constants Km and Vmax for rats and mice and to use the in vitro values of these parameters for scaling to the hamster and human where the same depth of in vivo data is not available. These values in conjunction with the known and measured physiological parameters for methylene chloride uptake and distribution will enable significant further development of the PB-PK model presented by Anderson et al (1987). The final objective of the CEFIC methylene chloride research programme is the development of a risk assessment based on these principles.

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