

**Method normalisation, validation protocols
and quality control for biomonitoring tests
for mutagens/carcinogens**

Eindverslag

**N° NM/01/32
NM/03/33
NM/10/34
NM/02/35**

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SUMMARY

A. Context

We know from animal studies that a large number of mutagens and carcinogens can be found in the workplace, the living environment, food and drinking water. Epidemiological methods up till now have only been able to prove conclusively the carcinogenic properties for humans of a limited number of these, including cigarette smoking, certain occupational exposures, drugs and ionising radiation.

The use of suitable biological markers can contribute in several important ways to the advancement of cancer epidemiology and cancer prevention. Possible benefits are the availability of better exposure data in epidemiological studies, the use of biomarkers for early effects instead of clinical disease as end-points and a more solid basis for extrapolation from experimental animals to humans.

The development of better biomarkers will also greatly contribute to a better risk surveillance and risk control. As such they may become valuable tools in occupational and community medicine for monitoring exposures to mutagens/carcinogens and for screening for pre-clinical effects. Studies on the basis of these biomarkers should also prove useful for regulatory bodies in the implementation of adequate control measures.

B. Objectives

In this context the concrete objectives of this study were to develop and validate a number of biomonitoring methods. They belong to different categories of biomarkers, namely biomarkers for internal dose, for biologically effective dose and for early biological effects. More specifically, these methods are:

- Development and validation of a method to assess the urinary excretion of inorganic arsenic metabolites in man.
- The alkaline comet or single cell gel electrophoresis assay for the detection of DNA breaks, alkali labile sites, open repair sites and DNA-protein cross-links on a single cell level. This test measures primarily exposure and, secondarily, early biological effects
- The determination of N-terminal valine adducts to haemoglobin by means of a modified Edman degradation technique for epoxide forming mutagens. This test also measures exposure and the earliest biological effect.
- The micronucleus test a tool for the determination of damage to the chromosomes caused by ionising radiation or chemicals. This test also measures biological effects.

C. Results and conclusions

Ci. Inorganic arsenic metabolites

The determination in urine of inorganic arsenic (iAs), which is a human carcinogen, and its relevant metabolites (MMA: monomethylarsonic and DMA : dimethylarsinic acids) allows to characterise an exposure to this element. Before using such a biomonitoring method, analytical difficulties must be resolved particularly with regard to the existence of two valence states (iAs^{III} and iAs^V) and organic species, some of which derive from biotransformations after absorption (MMA and DMA) and are to be considered in the assessment of exposure, and others from the ingestion of arsenicals present in seafood (AsB : arsenobetaine and AsC : arsenocholine), generally atoxic and to be disregarded for the interpretation.

A technique for the biomonitoring of exposure to iAs is developed: high pressure liquid chromatography on ion exchanger is selected to guarantee arsenic species discrimination; a prior hydride generation step makes it possible to avoid interferences from seafood arsenicals and an atomic fluorescence detector provides sensitivity and specificity at a low cost. A fully automatic technique is developed by coupling the three operations. In addition, conditions are defined to allow the on-line mineralization of AsB and AsC when their determination is wanted.

Starting from 50 µl of urine (which can be stored at 4°C for several weeks without loss), concentrations of the 4 species iAs^{III}, iAs^V, MMA and DMA as low as 1 µg As/L can be measured ; CV of replicate determinations amounts to 10% at the 2.5 µg As/L level and remains below 5% for concentrations equal or above 10 µg As/L.

Analytical performances of the automatic technique developed are similar to those of the previously used manual method based on atomic absorption. An excellent proficiency in the determination of the urinary As excretion is demonstrated by the results obtained in the course of interlaboratory comparison programs organized by German and Canadian institutes.

DMA measurements in standard materials (tuna fish extract and urine) lead to results in the tolerance range but are in one case below and in the other above the target value, pointing to the need for certified standard solutions of both MMA and DMA.

The usefulness of urinary As measurements is demonstrated in cases of acute (attempted suicide) and chronic (Spa water treatment of asthmatic boys) exposures to the element.

Cii. Styrene adducts

Many carcinogenic compounds cause mutations by chemically binding to DNA. The results of such binding processes are called adducts. Adducts may cause mutations because at the binding site copying errors may arise when DNA replication takes place during cell division.

The study of adducts is a valuable new tool in cancer risk assessment. Adducts may offer information on the mechanisms of mutation, the level of exposure of individuals to carcinogens and the degree of cancer risk. However, the direct study of DNA adducts in humans is difficult for a number of reasons. Therefore, indirect methods are sought that can serve the same purposes. For this reason adducts to proteins are frequently used as surrogate for DNA, because they are formed by the same chemical processes that lead to DNA adduct formation.

The study of adducts requires the development of extremely sensitive analytical techniques. The normalisation program offers a framework for research on method development and validation. The concrete aim was to develop and validate a method

for the determination of adducts to the blood protein haemoglobin. As model compound styrene was chosen. This substance is important for human health because of its widespread use in the plastics industry. It is also a putative mutagen because of its metabolism into styrene-7,8-oxide, a known mutagen and carcinogen.

The method developed consists of a sample preparation step allowing the specific isolation of adducts to the amino acid at the end of the haemoglobin chain (N-terminal valine), followed by a gas chromatographic and mass spectroscopic analysis. In the development stage rigorous procedures had to be devised to eliminate interference by other compounds both from within the sample and from external sources. The method was validated with respect of the required limit of detection; the linearity and fit of the calibration curve, the precision and accuracy (versus a certified commercial adduct standard). It was also shown in a group of workers exposed to low levels of styrene that the method is able to discern between adduct levels at the limit of detection and the background noise in a non-exposed reference group.

Ciii. Comet assay and micronucleus test

The main goal of the Laboratory for Cell Genetics was the validation of the micronucleus test and the Comet assay for the evaluation of DNA damage after exposure to mutagens. Practically, the laboratory's activities concentrated on the implementation of an internal standard in the Comet assay, the use of more specific reporter cells in both the Comet assay and the micronucleus test, the use of the Comet assay in a Global Repair Phenotype assay, the use of the micronucleus test in biomonitoring studies and some final discussions necessary for the validation and the international acceptance of the *in vitro* micronucleus test.

Implementation of an internal standard in the Comet assay

The electrophoresis step in the Comet assay is the most critical step of the method and is very sensitive to experimental variation. This can be minimized by the systematic use of an internal standard in every electrophoresis run. Our laboratory introduced and validated untreated and ethylmethane sulphonate treated K562 cells as an internal standard in the Comet assay.

Use of more specific reporter cells in both the Comet assay and the micronucleus test

In human biomonitoring studies the cells most commonly used are peripheral blood lymphocytes in the *ex vivo/in vitro* cytokinesis blocked micronucleus test (CBMN test) or in the regular *in vivo* micronucleus test, for the simple reason that peripheral blood lymphocytes are easy to obtain and to cultivate. Very often, these peripheral blood lymphocytes are actually not the target cells of certain chemicals, nor the cells being exposed to them directly. Nasal or buccal mucosal cells or lung epithelial cells in sputum for instance are the cells exposed directly when exposure through inhalation is considered (relevant for instance in the case of exposure to chemicals like chromium, cobalt and styrene). For this reason the laboratory tried to use these cells in both the Comet Assay and Micronucleus test.

Use of the Comet assay in a Global Repair Phenotype assay

Since more than 100 enzymes are involved in DNA repair along at least 5 different pathways, it is quite difficult to predict the repair phenotype from a selected number of DNA repair genotypes. By *in vitro* challenging with the involved mutagen, one can evaluate the *in vivo* repair phenotype.

The alkaline version of the Comet assay can be used for this *in vitro* evaluation of individual repair capacity e.g. for ionizing radiation or styrene (as was done in the laboratory), following the DNA damage with time (15', 30', 60', 12h and 24h).

Use of the micronucleus test in biomonitoring studies

Plenty of literature has been published on the cytokinesis blocked micronucleus test, but before strongly recommending this method it was essential to evaluate both its' negative and positive features. Fenech and co-workers summarized these. Nevertheless, until now, nobody took the presence of micronuclei in mononucleated cells into account, although it can be very complementary to the information embodied in binucleated cells. Later, Kirsch-Volders and Fenech suggested the integration of the frequency of micronuclei in mononucleated cells in the CBMN test in biomonitoring studies. This contribution will improve the assessment of accumulated mutations.

Towards validation and international acceptance of the in vitro micronucleus test

Micheline Kirsch-Volders was chairman of two International Workshops on Genotoxicity Testing (IWGT) (the 1999 IWGT in Washington and the 2002 IWGT in Plymouth). In these workshops the major topics of the test were discussed, the main goal being the recommendation of a protocol for a reliable *in vitro* micronucleus test for the detection of both clastogens and aneugens. The 1999 workgroup focused their discussion on the first step (the detection of micronuclei), and the 2002 workgroup had data available to prepare final conclusions on the main aspects of the *in vitro* micronucleus test protocol.

Civ. Micronucleus test

The determination of the micronucleus frequency in peripheral blood lymphocytes after *in vitro* mitogen stimulation is a valuable assay to assess the chromosomal damage in individuals exposed to ionising radiation or mutagenic chemicals. As different protocols for the test and a variety of scoring criteria are used throughout the world, normalisation and validation is necessary. The test is used as exposure biomarker in case of accidental, occupational, environmental and medical exposures. To give the test a legal dimension for regulatory bodies a quality assurance programme for accreditation of laboratories applying the micronucleus test was worked out.

In the framework of present project a standard protocol or *modus operandi* for the test was elaborated. The effect of different variables (culture time, fixation and staining methods) on the sensitivity and quality of the slides to be scored was investigated. A consistent set of scoring criteria was worked out in collaboration with the Human MicroNucleus working group, which aims the normalisation of the test at a world scale.

As a first step in the validation programme the reproducibility of the test was assessed by a study of the intra-laboratory variation in replicate cultures. Furthermore, the inter- and intra-individual variation of the test over nine months after an *in vitro* exposure of blood samples was studied. The intrinsic variability of the test amounts to 5 % while the intra- and inter-individual variabilities are 9 %.

The sensitivity of the micronucleus assay after a mutagenic exposure of an individual is determined to a large extent by the variability in the spontaneous micronucleus

frequency within a non-exposed population. A study of the micronucleus frequency distribution in a control population showed that age and gender are important confounding factors while the effect of smoking habit was statistically not significant.

Procedures were worked out for quality assurance and quality control of the micronucleus assay as exposure biomarker for mutagens. Also important elements in the validation file for the accreditation of laboratories performing the micronucleus assay were worked out: confidentiality of personal information, laboratory safety requirements, calibration curve, scoring procedure, reporting of results. These quality assurance and control programmes must assure the quality of the laboratory's output over extended periods of time. The elaboration of these procedures concurred with the draft of a document of the International Standardisation Organisation "Standard criteria for service laboratories performing biological dosimetry by cytogenetics- (Quality assurance and control, evaluation of performance) ".

D. Contribution of the project in a context of support to the processes of standardisation and technical regulations

Di Results directly connected with the process of normalisation

- The validation results of test methods that may contribute to the development of normalised testing protocols.
- The project allowed working out criteria for accreditation of laboratories performing cytogenetic analysis of blood samples for assessment of chromosomal damage after exposure to mutagenic chemicals and ionising radiation.

Dii Contacts with national and international institutions for normalisation

The following organisations were represented in the follow-up committee of the project:

- Beltest
- Ministry of Labour
- The Belgian Institute for Normalisation

Members of the project are participating in the following normalisation activities:

- At CEN: membership of Technical Committee 137 working on "Measurement of Dermal Exposure – Requirements and Test Methods. (KUL)
- Membership of Technical Committee 85 at the International Standardisation Organisation working on "Reference radiations" and "Physical and biological dosimetry". (RUG)
- Chairperson in the workshops on the in vitro micronucleus test at the IWGT held in Washington (1999) and Plymouth (2002). The proposed protocol will be integrated by OECD. (VUB)
- Coordination of the HUMN-project (HUMAN MicroNucleus project).(VUB)
- Evaluation for WHO-IPCS (World Health Organization – International Programme on Chemical Safety) of nitro-PAHs. (VUB)

Diii Presentation of the results to institutes for normalisation

- The results of the project with respect to the micronucleus assay are partially included in the draft document ISO/TC 85/SC 2 n 551 on "Standard criteria

for service laboratories performing biological dosimetry by cytogenetics- (Quality assurance and control, evaluation of performance“, now distributed for voting to the national members of the Technical Committee 85 of the ISO.

E. Keywords

Mutagens – Carcinogens – Biomarkers – Mutagenicity testing/DNA damage - In vitro/in vivo - Biomonitoring – Inorganic arsenic - Urinary metabolites determination - Haemoglobin adducts – Comet assay - Micronucleus test - Quality control

SAMENVATTING

A. Context

Uit dierstudies weten we dat een groot aantal mutagenen en carcinogenen aangetroffen worden op de werkplaats, in het leefmilieu, in voedsel en in drinkwater. Epidemiologische onderzoeksmethoden hebben tot op heden slechts voor een beperkt aantal daarvan onomstotelijk de carcinogene eigenschappen voor de mens kunnen aantonen. Daartoe behoren het roken van sigaretten, bepaalde beroepsblootstellingen en ioniserende straling.

Het gebruik van geschikte biologische merkers kan op verschillende belangrijke manieren bijdragen tot de vooruitgang in de epidemiologie en preventie van kanker. Tot de mogelijke voordelen behoren de beschikbaarheid van betere blootstellinggegevens in epidemiologische studies, het gebruik van biomerkers voor vroegtijdige effecten in de plaats van klinische ziektebeelden als eindpunten en een meer solide basis voor extrapolatie van proefdiergegevens naar de mens.

De ontwikkeling van betere biomerkers zal ook bijdragen tot een betere bewaking en beheersing van de het risico. Zij kunnen waardevolle hulpmiddelen worden in de arbeids- en milieugeneeskunde voor de bewaking van blootstellingen aan mutagenen/carcinogenen en voor het opsporen van preklinische effecten. Studies op de basis van deze biomerkers zullen ook nuttig blijken voor de regelgevende instanties voor de implementatie van aangepaste beheersingsmaatregelen.

B. Objectieven

In de bovenstaande context waren de concrete doelstellingen van deze studie de ontwikkeling en validering van een aantal methoden voor biomonitoring. Deze behoren tot verschillende categorieën van biomerkers, namelijk biomerkers voor interne dosis, voor biologisch effectieve dosis en voor vroegtijdige biologische effecten. Meer specifiek betreft het de volgende methoden:

- Ontwikkeling en validering van een methode voor de bepaling van metaboliëten van anorganisch arseen bij de mens.
- De *alkaline comet* of *single cell gel electrophoresis assay* voor de detectie van DNA-breuken, *alkali labile sites*, *open repair sites* en verbindingen tussen DNA en proteïne op het niveau van één enkele cel. Deze test meet hoofdzakelijk blootstelling en – secundair – vroegtijdige biologische effecten.
- De bepaling van N-terminale valine-adducten op hemoglobine met behulp van een gemodificeerde Edman-degradatietechniek voor epoxidevormende mutagenen. Deze test meet ook blootstelling en meest vroegtijdige biologische effect.
- De micronucleustest als hulpmiddel voor de bepaling van schade aan de chromosomen veroorzaakt door ioniserende straling of chemicaliën. Deze test meet ook biologische effecten

C. Resultaten en besluiten

Ci. Anorganische arseenmetabolieten

De bepaling in urine van anorganisch arseen (iAs), een humaan carcinogeen, en zijn relevante metabolieten (MMA: monomethylarsonzuur and DMA: dimethylarsinzuur) laat toe de blootstelling aan dit element te karakteriseren. Vooraleer een dergelijke biomonitoringmethode gebruikt kan worden moeten er eerst analytische problemen opgelost worden, in het bijzonder m.b.t. het bestaan van twee valentiestaten (iAs^{III} and iAs^V) en organische soorten, waarvan sommige afkomstig zijn van biotransformaties na de opname (MMA en DMA) en in aanmerking moeten worden genomen bij de bepaling van de blootstelling, terwijl andere afkomstig zijn van de ingestie van arseenverbindingen aanwezig in zeevoedsel (AsB: arsenobetaine en AsC: arsenocholine). Deze laatste zijn over het algemeen niet toxisch en dienen niet meegerekend te worden bij de interpretatie.

Een techniek voor de biomonitoring van blootstelling aan iAs werd ontwikkeld: hoge druk vloeistofchromatografie op een ionenwisselaar werd gekozen om selectief soorten van arseenverbindingen te kunnen onderscheiden. Een voorafgaande stap met vorming van hydride laat toe interferentie te voorkomen door arseenverbindingen uit zeevoedsel. Een atomaire fluorescentiedetector verschaft sensitiviteit en specificiteit aan een lage kost. Een volledig automatische techniek werd ontwikkeld door de drie operaties aan elkaar te koppelen. Bovendien werden de condities bepaald voor de *on-line* mineralisatie van AsB en AsC voor het geval hun bepaling gewenst is.

Vertrekkende van 50 µl urine (die gedurende meerdere weken bewaard kan worden zonder verlies bij 4°C) kunnen concentraties van de 4 soorten arseenverbindingen, iAs^{III}, iAs^V, MMA en DMA, tot 1 µg As/L worden gemeten. De CV van replicaatbepalingen bedraagt 10% voor een gehalte van 2.5 µg As/L en is lager dan 5% voor concentraties gelijk aan of hoger dan 10 µg As/L.

De analytische performantie van de geautomatiseerde techniek is vergelijkbaar met die van de vroegere manuele methode gebaseerd op atomaire absorptie. De resultaten bekomen tijdens interlaboratoriumvergelijkingen georganiseerd door Duitse en Canadese instituten hebben de validiteit van de methode aangetoond.

DMA metingen in standaardmaterialen (extract van tonijn en urine) leiden tot resultaten in de tolerantierange maar zijn in het ene geval onder en in het andere geval boven de gezochte waarde, hetgeen wijst op de nood aan gecertificeerde standaardoplossingen van MMA en DMA.

De bruikbaarheid van urinaire As-metingen werd aangetoond in gevallen van acute (zelfmoordpoging) en chronische (behandeling van astmatische jongens met Spa-water) blootstellingen aan het element.

Cii. Styreenadducten

Vele carcinogene stoffen veroorzaken mutaties door chemisch te binden met DNA. De producten van dergelijke bindingsprocessen worden adducten genoemd. Adducten kunnen mutaties geven omdat er op de plaats van de binding kopieerfouten kunnen optreden telkens DNA-replicatie plaatsvindt tijdens celdeling.

De studie van adducten is een waardevol hulpmiddel in de beoordeling van het kankerrisico. Adducten kunnen informatie verschaffen over het mutatiemechanisme, de graad van blootstelling van personen aan carcinogenen en de grootte van het kankerrisico. Het rechtstreeks bestuderen van DNA-adducten is moeilijk om een aantal

redenen. Daarom wordt gezocht naar indirecte methoden die voor dezelfde doelstellingen kunnen worden gebruikt. Om deze reden worden adducten met proteïnen dikwijls gebruikt als surrogaat voor DNA omdat zij gevormd worden door dezelfde chemische processen die leiden tot de vorming van DNA-adducten.

Het onderzoek naar adducten vereist de ontwikkeling van extreem gevoelige analytische technieken. Het normalisatieprogramma biedt een kader voor onderzoek naar methode-ontwikkeling en –validering. Het concrete doel was de ontwikkeling en validering van een methode voor de bepaling van adducten van het bloedproteïne hemoglobine. Als modelverbinding werd styreen verkozen. Deze stof is belangrijk voor de gezondheid van mensen omwille van haar wijdverspreid gebruik in de kunststofindustrie. Styreen is ook een vermoedelijk mutageen omwille van het metabolisme tot styreen-7,8-oxide, een erkend mutageen en carcinogeen.

De ontwikkelde methode omvat als eerste stap een staalvoorbereiding die toelaat adducten van het aminozuur aan het einde van de hemoglobineketen (N-terminaal valine) specifiek af te scheiden. Deze stap wordt gevolgd door een gaschromatografische en massaspectroscopische analyse. In de ontwikkelingsfase dienden er rigoureuze procedures op punt te worden gesteld om interferenties te elimineren door andere stoffen, zowel afkomstig van het staal als van externe bronnen. De methode werd gevalideerd m.b.t. de vereiste detectielimiet, de lineariteit en fit van de ijklijn, de precisie en accuraatheid (d.m.v. een gecertificeerde commerciële adductstandaard). Er werd ook aangetoond in een groep werknemers met lage blootstelling aan styreen dat de methode in staat is een onderscheid te maken tussen adductniveaus nabij de detectielimiet en de achtergrondruis in een niet blootgestelde populatie.

Ciii. Comet assay en micronucleustest

Het hoofddoel van het Laboratorium voor Celgenitica was de validering van de micronucleustest en de *comet assay* voor de evaluatie van DNA-schade na blootstelling aan mutagenen. In de praktijk waren de activiteiten van het laboratorium gericht op de implementatie van een interne standaard in de *comet assay*, het gebruik van meer specifieke reporter cellen in zowel de *comet assay* als de micronucleustest, het gebruik van de *comet assay* in een Globaal Repair Fenotype Assay, het gebruik van de micronucleustest in biomonitoringstudies en enkele finale discussies nodig voor de validering en de internationale aanvaarding van de *in vitro* micronucleustest.

Implementatie van een interne standaard in de Comet assay

De electroforesestap in de *comet assay* is de meest kritische stap van de methode en is zeer gevoelig aan experimentele variatie. Dit kan tot een minimum worden herleid door het systematisch gebruik van een interne standaard bij elke electroforese. Ons laboratorium introduceerde en valideerde onbehandelde en met ethylmethaansulfonaat behandelde K562 cellen als een interne standaard in de *comet assay*.

Gebruik van meer specifieke reporter cellen in zowel de comet assay als de micronucleustest

De meest gebruikte cellen in biomonitoringstudies bij de mens zijn perifere bloedlymfocyten in de *ex vivo/in vitro* cytokinese geblokkeerde micronucleustest (CBMN test) of in de gewone *in vivo* micronucleustest, om de eenvoudige reden dat perifere bloedlymfocyten gemakkelijk te bekomen en te cultiveren zijn. Zeer vaak zijn

deze perifere bloedlymfocyten niet de doelwitcellen van bepaalde chemicaliën, noch de cellen die er rechtstreeks aan blootgesteld zijn. Neuscellen, mond mucosacellen of longepitheelcellen in sputum zijn wel direct blootgesteld wanneer het blootstelling door inhalatie betreft (relevant bijvoorbeeld in het geval van chroom, kobalt en styreen). Daarom heeft het laboratorium gepoogd deze cellen zowel te gebruiken in de *comet assay* als de micronucleustest

Gebruik van de comet assay in een Globaal Repair Fenotype assay

Aangezien meer dan 100 enzymen betrokken zijn bij *DNA repair* langs minstens 5 verschillende *pathways* is het bijzonder moeilijk een *repair* fenotype te voorspellen op basis van een select aantal *DNA repair* genotypen. Door een *in vitro* blootstelling aan het betrokken mutageen kan men het *in vivo repair* fenotype evalueren.

De alkaline versie van de *comet assay* kan worden gebruikt voor deze *in vitro* evaluatie van de individuele *repair* capaciteit, bijvoorbeeld voor ioniserende straling of styreen (zoals gedaan in het laboratorium) waarbij de DNA-schade in de tijd werd gevolgd (15', 30', 60', 12h en 24h).

Het gebruik van de micronucleus test in biomonitoringstudies

Veel van de gepubliceerde literatuur is gewijd aan de cytokinese geblokkeerde micronucleustest, maar vooraleer deze methode sterk aan te bevelen was het essentieel zowel haar positieve als negatieve kenmerken te evalueren. Fenech en medewerkers hebben deze samengevat. Tot op heden bracht echter niemand de aanwezigheid van micronuclei in cellen met één kern in rekening, alhoewel dit zeer complementair zou kunnen zijn met de informatie verschaft door cellen met dubbele kern. Later hebben Kirsch-Volders en Fenech de integratie voorgesteld van de frequentie van micronuclei in éénkernige cellen in de CBMN test in biomonitoringstudies. Deze bijdrage zal de beoordeling verbeteren van geaccumuleerde mutaties.

Naar een validering en internationale aanvaarding van de in vitro micronucleustest

Micheline Kirsch-Volders was voorzitter van twee internationale workshops over getoxiciteitstesting (IWGT) (de 1999 IWGT in Washington en de 2002 IWGT in Plymouth). In deze workshops werden de hoofdpunten van de test besproken, was het doel de aanbeveling van een protocol voor een betrouwbare *in vitro* micronucleustest voor de detectie van zowel clastogenen als aneugenen. In de 1999 werkgroep was de discussie geconcentreerd op de eerste stap (de detectie van micronuclei), en de 2002 werkgroep had gegevens ter beschikking om eindconclusies voor te bereiden over de hoofdaspecten van het *in vitro* micronucleustestprotocol.

Civ. Micronucleustest

De bepaling van de frequentie van micronuclei in perifere bloedlymfocyten na een *in vitro* stimulatie met mutageen is een waardevol hulpmiddel om de chromosoomschade te vast te stellen bij individuen blootgesteld aan ioniserende straling of mutagene chemicaliën. Aangezien er verschillende protocols voor de test en een veiligheid aan scoringscriteria worden gebruikt over de ganse wereld, is er nood aan normalisatie en validering. De test wordt gebruikt als biomarker voor blootstelling in het geval van accidentele, beroeps-, milieu- of medische blootstellingen. Om de test een legale dimensie te geven voor overheidsinstellingen

werd een programma voor accreditering van laboratoria die de micronucleustest toepassen uitgewerkt.

In het kader van het huidige project werd een standaardprotocol of *modus operandi* voor de test uitgewerkt. De invloed van verschillende variabelen (incubatietijd, fixatie en kleuringmethoden) op de gevoeligheid en kwaliteit van de te scoren preparaten werden onderzocht. Een set van consistente scoringscriteria werd uitgewerkt in samenwerking met de *Human MicroNucleus Working Group*, die een wereldwijde normalisatie van de test nastreeft.

In een eerste stap van het valideringsprogramma werd de reproduceerbaarheid van de test bepaald d.m.v. een studie van de intra-laboratoriumvariabiliteit in replicaten van culturen. Verder werden de inter- en intra-individuele variatie van de test over negen maanden bestudeerd na een *in vitro* blootstelling van bloedstalen. De intrinsieke variabiliteit van de test bedraagt % terwijl de intra- en inter-individuele variabiliteiten 9% belopen.

De gevoeligheid van de micronucleustest na een mutagene blootstelling van een individu wordt in grote mate bepaald door de variabiliteit van de spontane micronucleusfrequentie in een niet blootgestelde populatie. Een studie van de micronucleusfrequentiedistributie in een controlepopulatie toonde aan dat leeftijd en geslacht belangrijke *confounding* factoren zijn terwijl het effect van rookgewoonte statistisch niet significant was

Procedures werden uitgewerkt voor de kwaliteitsbewaking en –borging van de micronucleustest als biomarker voor blootstelling aan mutagenen. Ook werden er belangrijke elementen in het valideringsdossier voor de accreditering van laboratoria die de micronucleustest uitvoeren uitgewerkt: de betrouwbaarheid van persoonlijke gegevens, vereisten op het vlak van laboratoriumveiligheid, de ijklijn, de scoringsprocedure, rapportering van resultaten. Deze kwaliteitsbewakings- en kwaliteitsborgingsprogramma's moeten de kwaliteit verzekeren van de resultaten van het laboratorium over een lange tijdsperiode. De uitwerking van deze procedures viel samen met het ontwerp van een document van de *International Standardisation Organisation* "*Standard criteria for service laboratories performing biological dosimetry by cytogenetics - (Quality assurance and control, evaluation of performance)*".

D. Bijdrage van het project in een context van ondersteuning aan het proces inzake normalisatie en technische regelgeving

Di Resultaten rechtsreeks betrokken op het proces van normalisatie

- Valideringsresultaten van testmethoden die bij kunnen dragen tot de ontwikkeling van genormeerde testprotocollen.
- Het project liet toe criteria uit te werken voor de accreditering van laboratoria die cytogenetische analyses uitvoeren van bloedstalen voor de bepaling van chromosoomschade na blootstelling aan mutagene stoffen en ioniserende stralingen.

Dii Contacten met nationale en internationale instituten voor normalisatie

De volgende organisaties waren vertegenwoordigd in de opvolgingscommissie van het project:

- Beltest
- Ministerie van Arbeid
- Belgisch Instituut voor Normalisatie

Leden van de projectgroep nemen deel aan de volgende normalisatie-activiteiten:

- Bij CEN: lid van Technical Committee 137 in het kader van "Measurement of Dermal Exposure – Requirements and Test Methods. (KUL)
- Lid van Technical Committee 85 bij het International Standardisation Organisation in het kader van "Reference radiations" en "Physical and biological dosimetry". (RUG)
- Voorzitterschap in de workshops over de in vitro micronucleustest bij de IWGT in Washington (1999) en Plymouth (2002). Het voorgestelde protocol zal geïntegreerd worden door OECD. (VUB)
- Coordinatie van het HUMN-project (HUman MicroNucleus project) (VUB)
- Evaluatie voor WHO-IPCS (World Health Organization – International Programme on Chemical Safety) van nitro-PAHs. (VUB)

Diii Presentatie van de resultaten aan instituten voor normalisatie

- De resultaten van het project m.b.t. de micronucleustest zijn gedeeltelijk opgenomen in het ontwerpdocument ISO/TC 85/SC 2 n 551 over "Standard criteria for service laboratories performing biological dosimetry by cytogenetics- (Quality assurance and control, evaluation of performance", Het document is ter stemming voorgelegd aan de nationale leden van het Technical Committee 85 van ISO.

E. Trefwoorden

Mutagenen – Carcinogenen – Biomerkers – Mutageniciteitstesting/DNA-schade - In vitro/in vivo - Biomonitoring – Anorganisch arseneen – Bepaling van urinaire metabolieten – Hemoglobine-adducten – Comet assay - Micronucleustest - Kwaliteitsbewaking

RESUME

A. Contexte

Le caractère mutagène et/ou cancérigène d'un grand nombre d'agents présents en milieu professionnel, dans l'environnement général, les aliments et l'eau de boisson a pu être identifié grâce à l'expérimentation animale. Jusqu'à présent, les études épidémiologiques n'ont cependant été capables d'établir le pouvoir cancérigène pour l'homme que dans un petit nombre de cas, parmi lesquels on trouve la consommation de cigarettes, certaines expositions professionnelles, l'usage de certains médicaments et l'exposition aux radiations ionisantes.

L'utilisation de marqueurs biologiques appropriés est à même de contribuer de diverses façons aux progrès de l'épidémiologie et donc de la prévention du cancer : ils peuvent faciliter l'extrapolation à l'homme des résultats obtenus chez l'animal ; on peut en attendre, au cours d'études épidémiologiques, une meilleure appréciation de l'exposition ; ils permettent le recours à des biomarqueurs d'effets précoces plutôt qu'à des symptômes cliniques.

La mise au point de meilleurs biomarqueurs doit aussi contribuer à une meilleure surveillance et une meilleure gestion des risques. A ce titre ils peuvent devenir en santé communautaire et médecine professionnelle d'excellents outils pour la surveillance des expositions aux mutagènes/cancérigènes et le dépistage d'effets pré-cliniques. Les études mettant en œuvre de tels biomarqueurs devraient intéresser les instances législatrices chargées de l'instauration de mesures de protection adéquates.

B. Objectifs

Les buts des études menées dans le cadre présenté ci-dessus sont le développement et la validation de quelques méthodes de surveillance biologique faisant appel à des marqueurs de différentes catégories : des marqueurs de dose interne, de dose biologiquement active et des effets biologiques précoces. Plus précisément, ces méthodes consistent en :

- développement et validation d'une méthode pour la mesure de l'excrétion urinaire des métabolites de l'arsenic inorganique chez l'homme
- la mise au point du test de la 'comète' ou électrophorèse sur gel d'une cellule unique permettant la détection de cassures de l'ADN, de sites labiles en milieu alcalin, de sites de réparation ouverts et de liens croisés entre protéines et ADN. Ce test met en évidence principalement l'exposition et secondairement des effets biologiques précoces.
- la détermination d'adduits à la valine de l'extrémité N-terminale dans l'hémoglobine au moyen d'une technique de dégradation d'Edman modifiée pour la détection de mutagènes formant des liens de type époxy. Ce test permet d'apprécier l'exposition et les effets biologiques les plus précoces
- le test des micronoyaux pour la détermination des atteintes induites dans les chromosomes par les radiations ionisantes et des substances chimiques. Ce test met en évidence des effets biologiques.

C. Résultats et conclusions

Ci. Métabolites de l'arsenic inorganique

Pour évaluer l'importance d'une exposition à l'arsenic inorganique (iAs), un cancérigène humain, on peut déterminer l'excrétion urinaire de l'élément et de certains métabolites, les acides monométhylarsonique (MMA) et diméthylarsinique (DMA). Mais avant d'utiliser ce type de surveillance biologique il convient de résoudre certaines difficultés analytiques liées à l'existence pour l'arsenic de deux états de valence (iAs^{III} et iAs^V) et d'espèces formées par métabolisme; si les dérivés MMA et DMA doivent être mis en rapport avec une exposition à iAs, il n'en va pas de même avec des composés tels l'arsénobétaïne (AsB) et l'arsénocholine (AsC) qui peuvent être excrétés en grande quantité après consommation de nourriture d'origine marine.

Au cours de ce travail, une technique de biosurveillance de l'exposition à iAs a été développée. La chromatographie en phase liquide à haute pression sur échangeur d'ions a été sélectionnée pour réaliser la séparation des diverses formes d'arsenic ; une étape de génération d'hydrures permet de ne pas prendre en compte les composés d'origine marine et un détecteur de fluorescence atomique assure des mesures avec sensibilité et spécificité. Le couplage de ces trois étapes a permis l'automatisation complète de la procédure. En outre, si besoin était, des conditions permettant la minéralisation en ligne de AsB et AsC ont été développées.

A partir de 50 µl d'urine (pouvant être stockée sans perte durant plusieurs semaines à 4°C) des concentrations de l'ordre de 1 µg As/L peuvent être mesurées dans le cas des 4 espèces (iAs^{III}, iAs^V, MMA et DMA) ; le coefficient de variation de mesures répétées s'élève tout au plus à 10% au niveau de concentrations de l'ordre de 2.5 µg As/L et reste inférieur à 5% pour des concentrations de 10 µg As/L et plus.

Les performances de la technique automatique sont aussi bonnes que celles de la méthode basée sur l'absorption atomique utilisée précédemment. Les résultats obtenus au cours de programmes de comparaison interlaboratoires, organisés par des instituts allemand et canadien, démontrent la validité de la méthode. La mesure de DMA dans des standards certifiés (urine et extrait de thon) conduit à des résultats dans la gamme de valeurs acceptables mais sont dans un cas inférieur et dans l'autre supérieur à la valeur cible, ce qui pourrait démontrer la nécessité de disposer de solutions standards certifiées pour (MMA et) DMA.

La technique de mesure de l'excrétion urinaire d'iAs et de ses métabolites a permis de suivre l'évolution d'un cas d'intoxication massive (tentative de suicide) comme de mettre en évidence un cas d'intoxication sub-aiguë (cure thermique appliquée à un groupe de jeunes asthmatiques).

Cii. Les adduits dus au styrène

De nombreuses substances cancérigènes provoquent des mutations en se fixant chimiquement à l'ADN et réalisant ainsi des adduits. Ces adduits peuvent provoquer des mutations car là où ils sont fixés des erreurs peuvent se produire lors de la réplication de l'ADN qui accompagne la division cellulaire.

L'étude des adduits est une nouvelle approche intéressante pour l'établissement du risque cancérigène. Les adduits peuvent donner des informations sur les mécanismes de mutation, le niveau d'exposition des sujets aux cancérigènes et l'importance du risque de cancer. Pour plusieurs raisons cependant l'étude directe des adduits à l'ADN est difficile chez l'homme et des approches indirectes ayant les mêmes buts sont recherchées. C'est pourquoi les adduits aux protéines plutôt qu'à

l'ADN sont fréquemment utilisés ; ils sont d'ailleurs formés par des processus chimiques identiques à ceux qui conduisent à la formation d'adduits à l'ADN.

L'étude des adduits demande le développement de techniques analytiques extrêmement sensibles. Le programme de normalisation fournit le cadre de recherches pour le développement et la validation de telles techniques. C'est ce qui a été concrètement réalisé dans le cas de la détermination d'adduits à l'hémoglobine en sélectionnant le styrène comme molécule type. Cette substance a une grande importance en santé humaine car elle est très largement utilisée dans l'industrie des matières plastiques ; elle est potentiellement mutagène car elle est métabolisée en styrène-7,8-oxyde, mutagène et cancérigène connu.

La méthode développée consiste en une première étape de préparation de l'échantillon permettant l'isolement d'adduits sur un acide aminé terminal de l'hémoglobine (valine N-terminale) ; suivent alors une chromatographie en phase gazeuse et une analyse par spectrométrie de masse. Dans la phase de développement des procédures très strictes ont dû être élaborées pour éliminer les interférences d'autres composés provenant tant de l'échantillon que du milieu extérieur. La méthode a été validée en rapport avec la limite de détection requise ; les caractéristiques de la courbe d'étalonnage et la précision et exactitude des déterminations ont été évaluées grâce à un étalon commercial certifié. Il a été possible de discriminer, dans un groupe de travailleurs faiblement exposés au styrène, un niveau d'adduits proche de la limite de détection du signal de bruit de fond observé dans un groupe contrôle non exposé.

Ciii. Tests de la comète et des micronoyaux

L'objectif principal du Laboratory for Cell Genetics (VUB) a été la validation des tests de la comète et des micronoyaux pour l'évaluation des atteintes à l'ADN causées par l'exposition à des mutagènes. En pratique, les activités du laboratoire ont été centrées sur la mise en œuvre d'un standard interne pour le test de la comète, l'utilisation de cellules sentinelles plus spécifiques pour les tests de la comète et des micronoyaux, l'utilisation du test de la comète dans le cadre de l'évaluation du phénotype global de réparation et l'utilisation du test des micronoyaux pour effectuer une surveillance de type biologique. Le travail se termine par des réflexions nécessaires à la validation et la reconnaissance internationale du test *in vitro* des micronoyaux.

Mise en œuvre d'un standard interne pour le test de la comète

Dans le test de la comète, l'étape d'électrophorèse est la plus critique et la plus sensible aux fluctuations des conditions expérimentales. On peut y remédier par l'emploi systématique d'un standard interne à ajouter lors de chaque électrophorèse. Notre laboratoire a introduit et validé l'utilisation de cellules K562 contrôles et traitées à l'éthylméthanesulphonate comme standard interne dans le test de la comète.

Utilisation de cellules sentinelles plus spécifiques dans les tests des micronoyaux et de la comète

Les cellules le plus communément utilisées dans les études de biosurveillance chez l'homme au moyen du test classique *in vivo* des micronoyaux et de sa variante *ex vivo/in vitro* CBMN (cytokinesis blocked micronucleus test) sont les lymphocytes sanguins périphériques car ils sont faciles à obtenir et à cultiver. Très souvent, ces lymphocytes périphériques ne sont en fait pas les cibles de certaines substances chimiques, pas plus d'ailleurs que les cellules qui y sont exposées directement. En

cas d'exposition par inhalation (par exemple au chrome, au cobalt, au styrène) ce sont particulièrement les cellules des muqueuses nasale et buccale ou les cellules de l'épithélium pulmonaire récupérables dans les expectorations qui sont le plus exposées. C'est la raison pour laquelle le laboratoire a essayé d'utiliser ces cellules dans le test de la comète.

Utilisation du test de la comète dans le cadre de l'évaluation du phénotype global de réparation

Comme plus de 100 enzymes sont impliqués dans les processus de réparation de l'ADN qui suivent au moins 5 voies différentes, il est très difficile de prédire le phénotype de réparation à partir d'un nombre limité de génotypes de réparation de l'ADN. On peut cependant évaluer le phénotype de réparation *in vivo* après avoir pratiqué une exposition au mutagène *in vitro*. La version du test de la comète en milieu alcalin peut être utilisée pour cette évaluation *in vitro* de la capacité de réparation individuelle après exposition aux radiations ionisantes ou au styrène (pratiquée au laboratoire) : il s'agit de suivre les atteintes à l'ADN 0.25, 0.5, 1, 12 et 24 h après l'exposition au mutagène.

Utilisation du test des micronoyaux au cours d'études de surveillance biologique

Une littérature abondante traite du test CBMN mais il est essentiel, avant d'en recommander l'usage, d'en évaluer les avantages et inconvénients. Fenech et ses collaborateurs les ont résumés. Jusqu'à ce jour, cependant, personne ne considère la présence de micronoyaux dans les cellules mononucléées, alors que cette information peut être très complémentaire à celle fournie par les cellules binucléées. Par la suite, Kirsch-Volders et Fenech ont suggéré l'intégration de la fréquence des micronoyaux dans les cellules mononucléées lors de l'application du test CBMN au cours d'études de surveillance biologique. L'établissement d'une accumulation de mutations en sera facilité.

Perspectives de validation et de reconnaissance internationale du test in vitro des micronoyaux

Micheline Kirsch-Volders a présidé deux workshops internationaux d'évaluation de la génotoxicité (1999 IWGT in Washington and 2002 IWGT in Plymouth). Les aspects importants du test des micronoyaux y ont été discutés dans le but de recommander un protocole permettant la détection fiable des clastogènes et des aneugènes. En 1999 le groupe de travail a centré ses discussions sur la première étape du test (la détection des micronoyaux), et en 2002, le groupe a disposé de suffisamment de données pour émettre des conclusions finales concernant les aspects essentiels du protocole d'application *in vitro* du test des micronoyaux.

Civ. Le test des micronoyaux

La détermination de la fréquence des micronoyaux dans des lymphocytes sanguins périphériques après stimulation par un mitogène constitue un test intéressant pour apprécier les dégâts aux chromosomes chez des sujets exposés aux radiations ionisantes ou à des substances chimiques mutagènes. Comme différents protocoles et une diversité de critères de comptage sont utilisés de par le monde pour ce test, des opérations de normalisation et de validation s'avéraient nécessaires. Le test est utilisé comme bioindicateur d'expositions accidentelles, professionnelles, environnementales ou médicales. Pour donner au test une dimension légale auprès

des instances législatrices, un programme d'assurance de qualité a été mis au point, permettant ainsi l'accréditation des laboratoires pratiquant le test.

Dans le cadre du présent projet, nous avons développé un protocole standard pour le test, une manière de l'exécuter. Nous avons étudié les effets de différentes variables (temps de culture, méthodes de fixation et de coloration) sur la sensibilité du test et la qualité des lames à examiner. Un ensemble cohérent de critères de comptage a été dégagé en collaboration avec le groupe de travail Human MicroNucleus en charge de la normalisation du test à l'échelle mondiale.

Dans une première phase du programme de validation, la reproductibilité du test a été établie en examinant la variation intra-laboratoire lors de cultures répétées. En outre, pendant neuf mois, les variations inter- et intra-individuelles du test ont été étudiées après une exposition *in vitro* des échantillons de sang. La variabilité intrinsèque du test s'élève à 5% tandis que les variabilités intra- et inter-individuelles sont de 9%.

La sensibilité du test des micronoyaux en cas d'exposition d'un sujet à un agent mutagène est très largement influencée par la variabilité de la fréquence spontanée des micronoyaux au sein d'une population non exposée. Une étude de la distribution de la fréquence des micronoyaux dans un groupe de sujets contrôles a montré que l'âge et le sexe sont d'importants déterminants ; à l'inverse, les habitudes tabagiques n'exercent aucun effet statistiquement significatif.

Des procédures ont été élaborées pour garantir et contrôler la qualité du test des micronoyaux en tant que bioindicateur d'exposition aux mutagènes. Des éléments importants de la validation des opérations préalable à l'accréditation des laboratoires effectuant le test des micronoyaux ont été dégagés : confidentialité des informations personnelles, exigences de sécurité en laboratoire, droite d'étalonnage, procédure de comptage, présentation des résultats. Ces programmes d'assurance et de contrôle de qualité doivent garantir la qualité des performances des laboratoires pour de longues périodes. L'élaboration de ces procédures s'est montrée en conformité avec un document de travail de l'Organisation Internationale de Standardisation « Standard criteria for service laboratories performing biological dosimetry by cytogenetics- Quality assurance and control, evaluation of performance ».

D. Contribution du projet dans le cadre de l'aide aux activités relatives à la normalisation et aux réglementations techniques

Di. Résultats directement liés aux activités de normalisation

- Les résultats de validation des méthodes de tests qui peuvent contribuer au développement de protocoles normalisés
- Le projet a permis d'élaborer des critères pour l'accréditation des laboratoires effectuant dans le sang des analyses cytogénétiques pour évaluer les dommages aux chromosomes après une exposition à des substances chimiques mutagènes ou des radiations ionisantes.

Dii. Contacts avec les institutions de normalisation nationales et internationales

Les organisations suivantes ont été représentées dans le comité d'encadrement du projet:

- Beltest

- Ministère du travail
- Institut Belge de Normalisation

Des membres du projet ont participé aux activités de normalisation suivantes :

- Comité Technique 137 « Mesure de l'exposition par voie cutanée – Exigences et méthodes de test » (KUL en tant que membre)
- Comité Technique 85 « Radiations de référence » et « Dosimétries physique et biologique » (RUG en tant que membre)
- IWGT Workshops (en tant que présidence) à Washington (1999) et Plymouth (2002) ; le protocole proposé sera intégré par l'OECD. (VUB)
- Coordination du projet Human MicroNucleus (VUB)
- Evaluation des nitro-PAH pour WHO-IPCS (VUB)

Diii. Présentation de résultats aux instituts de normalisation

- Les résultats du projet en rapport avec le test des micronoyaux sont en partie inclus dans le document provisoire ISO/TC 85/SC 2 n 551 « Standard criteria for service laboratories performing biological dosimetry by cytogenetics – Quality assurance and control, evaluation of performance » distribué actuellement pour vote aux membres du Comité Technique 85 de l'ISO.

E. Mots-clés

Mutagènes – Cancérigènes – Biomarqueurs – Evaluation de la mutagénicité/ des atteintes à l'ADN- In vitro/in vivo – Arsenic inorganique – Mesure de métabolites urinaires – Adduits à l'hémoglobine – Test de la comète – Test des micronoyaux – Contrôle de qualité

GENERAL INTRODUCTION

The importance of biomonitoring methods.

Cancer is a multi-step process with a long lag period, which is induced by a combination of mutational and non-genetic changes. Among the mutations, only a small fraction is inherited and thus most of them are induced by exogenous environmental factors. Therefore it is considered that controlling environmental exposures to mutagens/carcinogens might prevent most of the cancers. To better understand the mechanisms responsible of cancer induction, to be able to classify the environmental mutagens/carcinogens and finally, on the basis of these data, to allow pertinent prevention, biomonitoring studies are essential.

However, up till now the conventional tools to identify and assess potential cancer risks have been largely deficient. Epidemiological methods have only been able to prove conclusively the carcinogenic properties for humans of a limited number of exposures, including cigarette smoking, certain occupational exposures, drugs and ionising radiation. All the agencies in charge of classifying carcinogens (EPA, IARC, EU) agree on the fact that an environmental compound can be recognised as a human carcinogen only if clear evidence from epidemiological data is provided. One exception was the classification of ethylene oxide as class 1 by IARC on the basis of very extensive genotoxicity data in vivo in animals and human biomonitoring studies.

On the other hand we know from animal studies that a much larger number of mutagens and carcinogens can be found in the workplace, the living environment, food and drinking water. This gap between what is known with certainty and what is still largely a matter of conjecture, will in the future only be filled with the aid of well designed biomonitoring programs in human populations.

The use of biological markers can contribute in several ways to the advancement of cancer epidemiology. It is expected that these markers will not only allow the early identification of potential risks to humans, but will also provide comparative dosimetry and response data, which will greatly benefit the following aspects of cancer research.

- The incorporation of reliable exposure tests in epidemiological studies will enhance the power of these studies to find relationships between exposures to specific agents and increased cancer risks by reducing the misclassification error. Misclassification of subjects with respect to their exposure (exposed versus non-exposed, high versus low exposed) is believed to be the main cause of false-negative results in human epidemiology.
- Likewise, certain biomarkers of pre-clinical response can ensure an important improvement by replacing clinical disease as an end-point. By providing an earlier and more commonly occurring outcome, such markers would mitigate the latency problem in cancer epidemiology, increase study power and allow effective intervention.
- Finally, more reliable data on dose and response in humans will also facilitate the quantitative extrapolation from experimental animals to humans. This will prove to be another important tool in the assessment of cancer risk, that at present is insufficiently exploited because of the tremendous uncertainties involved.

Apart from additional epidemiological research data, improved methods in biomonitoring will also result in a better risk surveillance and risk control.

- With respect to the first, the means available to occupational and community medicine to monitor exposure and effects of mutagens/carcinogens are at present very limited. More specific and sensitive methods will aid immensely to target populations who are at special risk.
- One aspect of cancer prevention is the control of risks through regulatory measures, which have to be implemented by the responsible governmental bodies, both on a national and supranational level. The importance of reliable biomonitoring tests in the latter context not only resides in the fact that these tests may be instrumental in proving cancer risks, but that they may also form the basis for establishing limit values for acceptable exposure to control these risks.

Types of markers in biomonitoring

Several categories of biological assays belong to the broad domain of biomonitoring. This project encompasses most types of laboratory tests that can be used to assess exposures to and effects from mutagenic/carcinogenic agents. Generally, the following subdivisions are made in this domain:

- Parameters for **internal dose** measure the amount of carcinogen or its metabolites in tissues, cells or body fluids. This type of assays is most useful in assessing the total uptake by multiple routes and/or from multiple sources.
- Parameters for **biologically effective dose** reflect the amount that has interacted with cellular macromolecules and are either directly or indirectly a measure for molecular lesions at the level of DNA.
- A third category is called **markers of (early) biological effect**. These markers indicate an (irreversible) effect that is known or believed to be linked to cancer.
- Finally, **markers of susceptibility** represent tests that reflect the individual predisposition to the effects of mutagens/carcinogens. This category lies beyond the scope of this proposal.

The biomonitoring methods developed in this study

In this study a number of biomonitoring methods were developed and validated to further the aims outlined above. They belong generally to the first three categories described in the previous paragraph. More specifically, these methods are:

- Development and validation of a method to assess the urinary excretion of inorganic arsenic metabolites in man.
- The alkaline comet or single cell gel electrophoresis assay for the detection of DNA breaks, alkali labile sites, open repair sites and cross on a single cell level. This test measures primarily exposure and, secondarily, early biological effects

- The determination of N-terminal valine adducts to haemoglobin by means of a modified Edman degradation technique for epoxide forming mutagens. This test also measures exposure and the earliest biological effect.
- The micronucleus test a tool for the determination of damage to the chromosomes caused by ionising radiation or chemicals. This test also measures biological effects.

For reasons of clarity the following sections contain a separate description of the main results and conclusions for these different tests.

PART I

DEVELOPMENT AND VALIDATION OF A METHOD TO ASSESS THE URINARY EXCRETION OF INORGANIC ARSENIC METABOLITES IN MAN.

1. INTRODUCTION

Inorganic arsenic (iAs) is a human carcinogen occurring in some occupational settings but also in the general environment (mainly in drinking water) (IPCS, 2001). To control this cancer risk, the existence and importance of the exposure to the element and, preferably, its total uptake (by multiple routes and/or from multiple sources) must be assessed. Such a task can be performed by the determination of the **internal dose** which reflects the amount of carcinogen or its metabolites in tissues or body fluids such as urine (Lauwerys and Hoet, 2001a).

The existence of different valence states (III and V) and type of As derivatives (inorganic and organic) requires specific analytical procedures to perform a correct assessment of the exposure to iAs, avoiding possible interferences due to non toxic arsenicals present in food from marine origin and taking into account metabolic methylations of the element in the human organism.

2. METHODOLOGY

2.1 Required characteristics of the technique to be developed

To determine the urinary excretion of iAs and its metabolites in man, i.e. inorganic trivalent (iAs^{III}) and pentavalent (iAs^V) forms, monomethylarsonic (MMA) and dimethylarsinic (DMA) acids (Buchet and Lauwerys, 1981), an analytical technique is to be developed combining automation with:

- specificity: human iAs metabolites have to be distinguished from seafood arsenicals (arsenobetaine AsB, arsenocholine AsC, ...)
- sensitivity: limits of detection must allow the determination of the basal urinary excretion in subjects without a particular exposure to the element
- reproducibility: the goal is to make measurements with a coefficient of variation < 10%
- accuracy: results of measurements in certified materials must lie in the acceptable range around the target value; however certified materials are scarce and not available for all As species in urine; human urine Certified Reference Material N°18 from the National Institute for Environmental Studies (Tsukuba, Japan) and BCR-627 tuna fish tissue from the Institute for Reference Materials and Measurements (Geel, Belgium) are used for DMA measurements.

2.2 Analytical technique outline

Ion exchange chromatography is used to separate the different arsenicals:

- some are directly reducible by $NaBH_4$ (iAs^{III} , iAs^V , MMA and DMA) to gaseous derivatives (arsines: AsH_3 , CH_3AsH_2 and $(CH_3)_2AsH$) which are detected by atomic fluorescence spectrometry (AFS); these species are those involved in the human iAs metabolism

- trimethylated arsenicals (AsB, AsC) must be mineralized by $K_2S_2O_8$ treatment and UV irradiation before the reduction step to allow arsine generation and its detection by AFS; trimethylarsine oxide (TMAO) which is not a iAs metabolite in man, is directly reducible by $NaBH_4$ but its detection is easier after mineralization.

The presence of trimethylated As species in urine samples, especially AsB, reflects seafood consumption which can sometimes also cause an increased urinary excretion of DMA. It is thus important to be able to relate an increased DMA excretion either to seafood consumption or exposure to iAs: in the first instance, AsB excretion is observed and the amounts of iAs^{III} , iAs^V and MMA remain in the basal range while, in the second, the excretion of trimethylated forms is null or negligible but the increased excretion of DMA is concomitant with that of the other metabolites relevant to the human metabolism of iAs (iAs^{III} , iAs^V and MMA).

Actually, efforts have been focused on the development of a fully automatic technique allowing the measurement of directly reducible arsenicals.

2.3 Apparatus

A Gilson 321 pump with two reciprocating pump motors is used to feed Hamilton ion exchange columns

- PRPX100, 25 cm, ID 4.1 mm, particle size $10\mu m$ to separate directly reducible arsenicals (iAs^{III} , iAs^V , MMA and DMA)
- PRPX200, 25 cm, ID 4.1 mm, particle size $10\mu m$ followed by a PRPX100, 10 cm, ID 4.1 mm, particle size $10\mu m$, to separate AsB, AsC and TMAO in addition to the directly reducible species.

Samples ($50\mu l$) are introduced with a Bio-Tek Kontron (Beun DeRonde, Drogenbos, Belgium) autosampler. The atomic fluorescence detector is from PSA analytical (Excalibur type, Orpington, Kent BR5 3HP, UK). The Perma Pure (Toms River, NJ 08754-2105) MDTM-Series gas dryer originally included in the AFS instrument has been replaced by a vertical refrigerated condenser placed on top of the gas-liquid separator. Data acquisition is performed with a Chemstation from Agilent Technologies Deutschland GmbH, 76337 Waldbronn, Germany).

The photooxidation reactor is equipped with a 8 W UV-C Sylvania lamp (Japan) or a 15 W low pressure Hg lamp (Heraeus Noblelight TNN 15/32) around which a 3 m Teflon tubing (ID 0.5 mm, OD 1.58 mm) is wrapped up allowing a 90 sec irradiation period.

Teflon tubing (ID 0.3 mm ; OD $\frac{1}{4}$ ") is used as transfer line. Admission of argon and hydrogen and addition of reagents ($NaBH_4$, $K_2S_2O_8$ and HCl) are made through 3-way 0.8mm bore Teflon connectors (Omnifit, Cambridge CB13HD, England). To optimally stabilize the hydrogen flow feeding the AFS detector flame a mass flowmeter Mass-Trak instrument from Sierra (Monterey CA 93940, USA) is used.

2.4 Reagents

Common laboratory reagents (p.a. grade) are purchased from Merck (64293 Darmstadt, Germany); solutions are prepared with ultrapure water from a Nanopure II Sybron Barnstead system (Dubuque, IA, 52004-0797, USA).

The origin and quality of arsenicals are as follows:

iAs^{III}: sodium meta-arsenite p.a. Merck Darmstadt Germany
 iAs^V: arsenic acid sodium salt heptahydrate Sigma-Chemie D-8024 Deisenhofen Germany
 disodium methylarsinate hexahydrate 99.4% Carlo Erba Milano Italy
 DMA: dimethylarsinic sodium salt trihydrate for synthesis 98% Merck Darmstadt Germany
 TMAO: trimethylarsine oxide monohydrate >98% Argus Chemicals 59023 Vernio Italy
 AsB: arsenobetaine calibrant solution EU BCR reference material # 626 1031±6 mg/kg
 AsC: arsenocholine >98% Argus Chemicals 59023 Vernio Italy

Standard stock solutions containing 1 mg As/ml are prepared in Nanopure water ; working standard solutions (range : 10-100 µg As/L Nanopure water) are prepared daily in polypropylene tubes (glass or polystyrene vials are not suitable).

2.5 External quality control

External quality control is performed by participation in two international interlaboratory comparison programs organized by:

- the German Society of Occupational Medicine and Environmental Health, Institute for Occupational, Social and Environmental Medicine, Schillerstrasse,2, 91054 Erlangen, Germany (Prof. Dr. H. Drexler)
2 urine samples at 2 different levels, twice a year; target values are given for total As (seafood As excepted) and for iAs^{III}, iAs^V, MMA and DMA, individually
- the Institut National de Santé Publique du Québec, 945 av. Wolfe, Sainte-Foy, Qc, Canada G1V 5B3 (Dr JP Weber)
3 urine samples, 6 times a year; original urine samples from control, occupationally exposed or seafood consumer and samples spiked with the different As forms are dispatched ; target values are given only for the sum of metabolites (iAs^{III}, iAs^V, MMA and DMA)

3 RESULTS

3.1 Selection of chromatographic conditions

iAs and its metabolites in man are polar compounds with strikingly different first acid dissociation constants: pK_a's for iAs^{III}, iAs^V, MMA and DMA are 9.2, 2.3, 3.6 and 6.3, respectively. At the opposite, arsenicals from marine origin most often involve trimethylarsonium groups positively charged at the physiological pH. While a single anion exchanger may suffice to separate iAs and its metabolites in man (directly reducible As forms), a combined use of anion and cation exchangers is required to discriminate between these arsenicals and those from seafood origin.

3.1.1 Separation of directly reducible arsenicals (iAs^{III}, iAs^V, MMA and DMA)

An anion exchanger made of a copolymer polystyrene-divinylbenzene substituted with tetramethylammonium groups is used as stationary phase (Hamilton PRPX100, length 25 cm, ID 4.1mm, particle size 10µm). The chromatographic separation is obtained by adjustment of the pH and ionic strength of the mobile phase. Phosphate buffers pH 6 0.01 M (A) and 0.1 M (B) are used: 100% A during 6min ; increase of B up to 40% in 4 min; staying 6 min at this concentration and back to 100% A during 4

min. Best results are obtained for an elution at a flow rate of 1 ml/min; retention times for iAs^{III} , iAs^V , MMA and DMA are 2.9, 4.8, 8.0 and 13.2 min, respectively.

3.1.2. Separation of iAs human metabolites and arsenicals from marine origin

When trimethylated arsenicals from seafood origin (present as cations in urine) are to be detected concomitantly with As species related to an exposure to iAs (present as anions in urine), an analytical solution is the use of both a cation and an anion exchanger in series. Numerous trials lead to use an Hamilton PRPX200 column (length 25 cm; ID 4.1mm; particle size 10 μm) placed in front of an Hamilton PRPX100 column (length 10 cm; ID 4.1mm; particle size 10 μm). Elution uses H_3PO_4 1mM (A) and 0.1 M PO_4 buffer pH 4; after 8 min with 100% A, up to 20% B is added in 30 min; 100% A during 12 min restores initial conditions.

Retention times for iAs^{III} , DMA, MMA, AsB, iAs^V , TMAO and AsC are 6.0, 9.6, 13.6, 22.2, 32.8, 34.8 and 36.8 min, respectively. It must be stressed that a mineralization step is necessary to detect trimethylated arsenicals by AFS. Optimization of oxidation conditions are presented below.

3.2 Optimization of the conditions of detection by atomic fluorescence spectrometry

Arsenic atomization is provided by combustion in a hydrogen flame of volatile arsines generated by reduction with $NaBH_4$ at pH between 0 and 1. As the hydrogen produced by the decomposition of $NaBH_4$ in acid medium is not sufficient to fuel the flame, an extra H_2 flow is to be provided. The arsine transfer to the gas phase is achieved by argon injections just after addition of the reducing agent (primary Ar flow) and in the gas-liquid separator (secondary Ar flow. An adjustment of several gas and liquid reagents flows is thus necessary to optimize the detector response. In addition, pre-reductive treatments of samples before reaction with $NaBH_4$ as well as parameters of the oxidation step needed for the measurement of non directly reducible arsenicals (trimethylated As) are to be considered.

3.2.1 Optimization of gases and liquid reagents flow rates

The 'signal to background ratio' (SBR) for a fixed concentration of each directly reducible As forms allows the comparison of the effects of changes induced by variations of the analytical conditions. As the reduction step needs a pH between 0 and 1, acidification of the buffered eluate before addition of the alkali stabilized $NaBH_4$ solution is performed by injection of 1ml/min of HCl 1.5 M (tentative choice; for adjustment see 3.2.3). On the basis of observations made with increasing Ar flow rates, the primary and secondary argon flows are fixed at 200 ml/min as for higher flow rates the H_2 flame is extinguished (it must be stressed that there is an upper limit for the H_2 flow otherwise the shape and position of the flame do no more fit in with the detector window. SBR changes due to variations of the two H_2 sources ($NaBH_4$ decomposition and pure gas addition) lead to the selection of 1.5%(w/v) $NaBH_4$ in NaOH 2%(w/v) and a H_2 flow rate of 70 ml/min as a compromise for the measurement of the 4 As species.

3.2.2 Usefulness of pre-reductive treatments

A prior reduction of pentavalent to trivalent state of As in iAs^V , MMA and DMA might enhance the generation of corresponding arsines. Addition of KI 10% (w/v) or cystein 10%(w/v) to the eluate acidifying HCl 1.5 M causes no signal increase. The addition of these reducing agents to samples 1 h before analysis is not more efficient.

3.2.3 pH adjustment of the reaction medium during arsine generation

The optimal molarity of HCl to add to the eluate before the reduction step is derived from observations in the 1 to 4 M range : the responses obtained for iAs^{III} and MMA do not seem change in the range of HCl concentrations studied but while the signal for iAs^V increases with the HCl molarity, the reverse behavior is observed with DMA. As a compromise a HCl concentration of 2 M is selected.

3.2.4 Selection of best conditions for the oxidation of trimethylated arsenicals

Trimethylated arsenicals such as AsB and AsC must be mineralized into iAs^V to generate a signal using AFS. A photo-oxidation of these components is performed by an on-line treatment of the eluate with an alkaline solution of $K_2S_2O_8$ coupled with a UV irradiation. Parameters influencing the mineralization step include $K_2S_2O_8$ concentration, medium alkalinity, UV source power and irradiation duration. After several assays, a Teflon coil of 3m in length, OD 1.58 mm and ID 0.5 mm was selected and allowed a 90 sec UV irradiation period by a 15 W UV lamp ; 1% (w/v) $K_2S_2O_8$ in 0.25 M NaOH proved to be best conditions for routine work.

3.3 Analytical performances

Many difficulties surfaced during this study, mainly in relation with variations between days and between As species. Major and minor causes were progressively unravelled and more or less satisfactorily attenuated : they included differences in the flow rates of gas or reagent solutions (modifying the detector flame quality) and, mainly, the aging of standard solutions of the As species.

3.3.1 Assessment of response linearity

Standard stock solutions of iAs^{III} , iAs^V , MMA and DMA containing 1g As/L, prepared in Nanopure water and diluted to have standard working solutions between 0 and 100 $\mu\text{g As/L}$ are used to verify the range of response linearity of the fluorescence signal (Fig. 1).

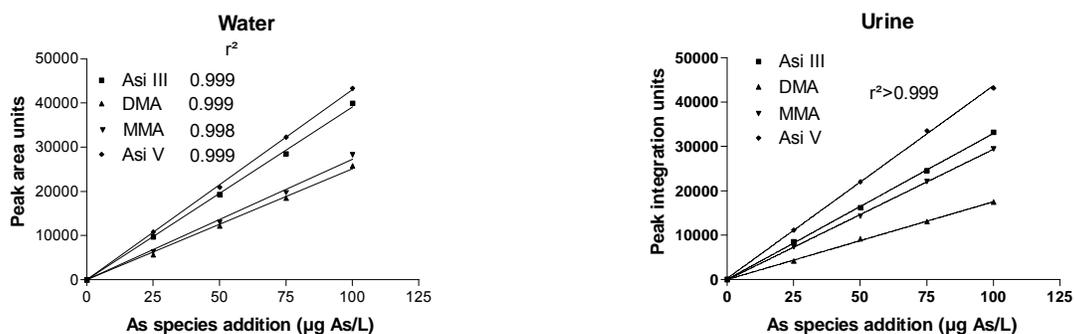


Fig. 1. Linearity of AFS detector response

Instrument calibration with standards in the range up to 100 $\mu\text{g As/L}$ in water as well as in urine generates linear relationships between signal and concentration ($r^2 > 0.99$). In a narrower concentration range in urine (between 0 and 10 $\mu\text{g As/L}$) linearity of response is still observed although observations are slightly more scattered ($r^2 > 0.95$).

3.3.2 Sensitivity and reproducibility determinations

Slopes of linear regression lines in Fig. 2 differ according to the As species considered: indeed, it is conceivable that the kinetics of reduction by NaBH₄ differ due to valence and methylation state. More surprising is the difference of slopes obtained in water and in urine. Eventually, it could be demonstrated that the working standard solutions of As species have a limited stability in water particularly in polystyrene plastic materials; polypropylene was selected and final dilutions of standard solutions prepared daily.

Using five different urine samples spiked with 25 and 50 µg As/L as iAs^{III}, iAs^V, MMA and DMA, it can be shown that a 'matrix effect' of urine can be considered as negligible; sensitivity (signal increase/concentration unit of As species) is highest for the inorganic forms; in water the sensitivity for iAs^{III} and iAs^V is almost the same but a partial oxidation of iAs^{III} into iAs^V can generally be observed when added to urine.

These internal standard additions allowed to assess routine detection limits in the range between 1 and 2 µg As/L in the case of the four As species when 50 µl sample are used for the determinations.

The reproducibility of determinations has been assessed by measurements of different urine samples spiked with 2.5 or 10 µg As as the four species (iAs^{III}, iAs^V, MMA and DMA). The relative standard deviation RSD (%) or the coefficient of variation CV (%) allows to compare measurements whatever the concentration level. However, according to Horwitz cited by Thompson and Lowthian (1997), a maximal acceptable level of intralaboratory variability, influenced by the concentration level can also be defined by :

$$CV (\%) = 2/3 * 2^{1-0.5\log C}$$

where C represents the ratio between the amounts of the analyte and its matrix. At the 2.5 and 10 µg/L levels, C=2.5*10⁻⁹ and 10*10⁻⁹, respectively. The following table gives, for both concentrations levels, values of observed CV (%) and the maximal acceptable variability according to the Horwitz rule :

	2.5 µg/L level				10 µg/L level			
	iAs ^{III}	iAs ^V	MMA	DMA	iAs ^{III}	iAs ^V	MMA	DMA
CV (%)	7	11	10	7	1	3	3	5
Horwitz	26				21			

3.4 Effects of sample pretreatments

Three sample cleaning procedures before the chromatographic step have been tested.

- The elimination of non polar compounds from urine samples was performed by using Sep-Pak C₁₈ cartridges. Almost all the total arsenic content of the samples was recovered and the elution profile of the chromatographic peaks corresponding to the four As metabolites was not improved.
- No improvement could be observed after samples centrifugation at 20 000 g*min.

- Protein precipitation by HClO_4 leads to an acidification of the sample, which cannot be sufficiently controlled by the elution buffer: the component separation power of the chromatographic column is lost.

Thus it was decided to analyse original urine samples and to protect the chromatographic column with a small C_{18} precolumn. In addition a column regeneration procedure was included in routine work: monthly, the chromatographic column was washed with 0.5% HNO_3 in methanol and water.

3.5 Study of sample storage conditions

In a paper published recently (Feldmann et al., 1999) authors concluded that "low temperature (4 and -20°C) conditions are suitable for the storage of urine samples for up to 2 months. Untreated samples maintain their concentration of arsenic species, and additives have no particular benefit". Therefore it was decided to check the stability of urine samples kept at 4°C only. Samples corresponding to the two last deliveries in 2002 by the Centre de Toxicologie du Québec (CTQ) in the frame of the international interlaboratory comparison programme were used (samples Q13, Q14 and Q15 were received in September; Q16, Q17 and Q18 in November 2002.)

In the following table, results obtained at one week interval are reported for each As species determined and their sum (which can be compared with the target value from CTQ).

Study of sample storage conditions

	Date of measurement					Mean	SD	CV (%)
	Dec 04	Dec 12	Dec 17	Dec 24	Jan 03			
Q13 ; ingestion of Asi III ; target : 393 $\mu\text{g As/L}$								
Asi III	60	59	55.5	52.5	55	56.4	3.1	5.4
DMA	235.5	259	269	245	262	254.1	13.6	5.3
MMA	133.5	143	161.5	133.5	126	139.5	13.7	9.8
Asi V	0	0	0	0	0			
As tot	458	465	486	431	446	457.2	20.6	4.5
Q14 ; addition of Asi V ; target : 56.2 $\mu\text{g As/L}$								
Asi III	32.9	31.1	32.6	29.6	31.8	31.6	1.3	4.2
DMA	0	0	0	0	0			
MMA	0	0	0	0	0			
Asi V	22.1	25.4	25.5	22.3	25.2	24.1	1.7	7.2
As tot	56.8	56.5	58.4	51.9	57.6	56.2	2.5	4.5
Q15 ; addition of DMA ; target : 26.2								
Asi III	0	0	0	0	0			
DMA	30.2	32.8	33.2	31.4	34.6	32.4	1.7	5.2
MMA	0	0	0	0	0			
Asi V	0	0	0	0	0			
As tot	30.2	32.8	33.2	31.4	34.6	32.4	1.7	5.2
Q16 ; exp. worker urine ; target : 199 $\mu\text{g As/L}$								
Asi III	54.8	55.2	59.2	50.2	53.4	54.6	3.3	6.0
DMA	123.2	133.4	144.4	124.8	143.8	133.9	10.1	7.5
MMA	11	9	12	11	16.4	11.9	2.8	23.2
Asi V	3.8	4.2	4.6	3.4	2	3.6	1.0	27.8

As tot 192.8 201.8 220.2 189.4 215.6 204.0 13.6 6.7

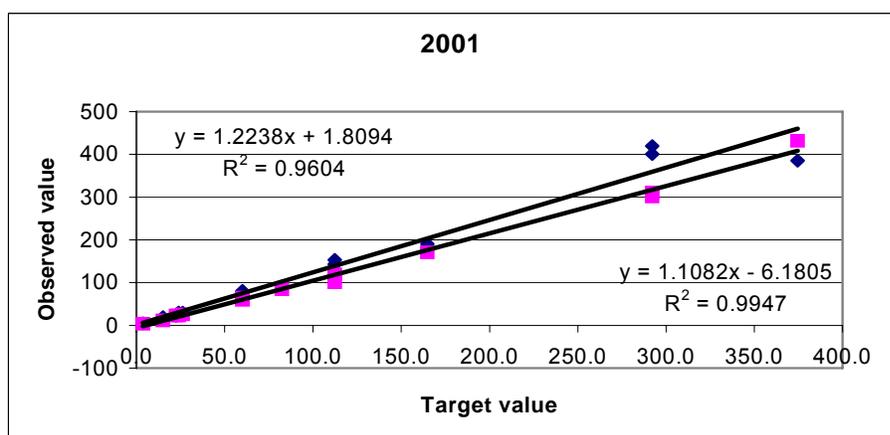
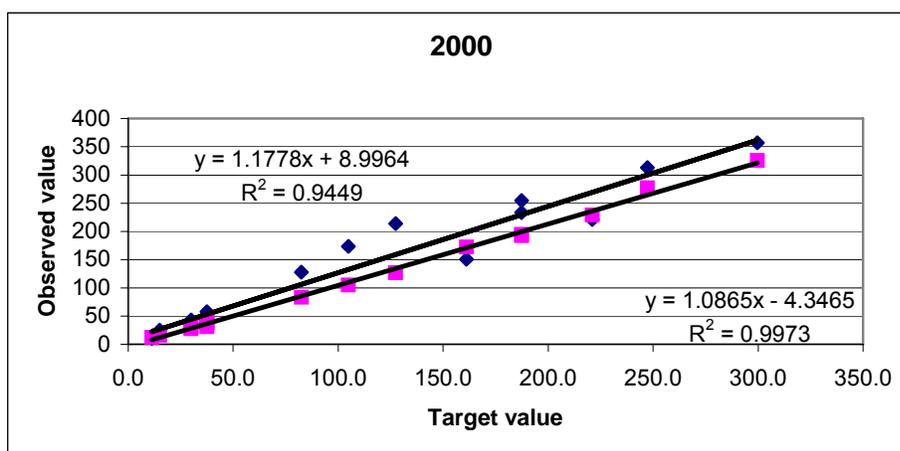
Q17 ; addition of MMA ; target : 52.4 µg As/L

Asi III	0	0	0	0	0			
DMA	0	0	0	0	0			
MMA	63	54.6	58.2	54.5	47.5	55.6	5.7	10.2
Asi V	0	0	0	0	0			
As tot	63	54.6	59	55.2	50.1	56.4	4.9	8.6

Q18 ; addition of DMA ; target : 97.4 µg As/L

Asi III	0	0	0	0	0			
DMA	118	116.1	130.5	116	136.2	123.4	9.4	7.6
MMA	0	0	0	0	0			
Asi V	0	0	0	0	0			
As tot	118	116.1	130.5	116	136.2	123.4	9.4	7.6

Results show that even after a 4 month storage at 4°C there is no trend with time in the concentration values. Urine samples from Québec received in 2000 and 2001 were also analyzed in the second part of 2002, after a much longer storage period at 4°C (up to 2 years). The correlation between measurements and target values is depicted by the upper straight line in the 3 graphs shown in Figure 2; R² values indicate a slight deterioration of the correlation with prolonged storage time.



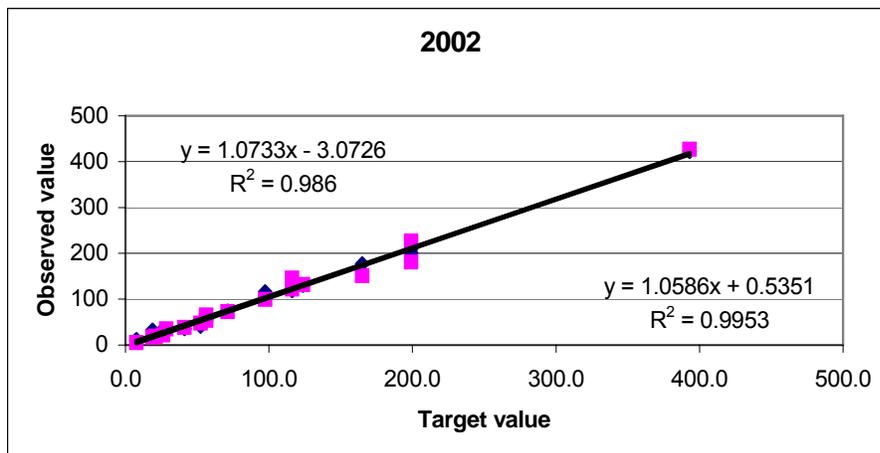


Fig.2. Analyses of Québec samples

The lower straight line in each graph of Fig. 2. gives the agreement between target values and measurements, without the chromatographic step, of the sum of As_i, MMA and DMA (all species are measured together).

This analysis procedure actually corresponds to the routine measurement in our laboratory of As excretion as a biomarker of exposure to the inorganic form of the element. In 2000 and until mid-2001, detection of 'directly reducible As forms' has been performed by atomic absorption spectrometry (AAS). Thereafter, atomic fluorescence spectrometry (AFS) was used.

When ion-exchange chromatography is not used, instrument calibration can be performed with acidified standard solutions which prove to be very stable but the detectors used for AAS as well as for AFS display different sensitivities for equimolar solutions of the different species taken separately. Instrument calibration is thus performed using a mixture of As species in proportions similar to those present in the samples to be analyzed. Actually, a solution in HCl 1.25 M containing 12.5, 12.5, 10 and 50 µg As/L as As_i^{III}, As_i^V, MMA and DMA, respectively, is used. As shown by the lower lines in graphs of Fig. 4, the 'direct' technique slightly overestimates the As concentrations.

Turning back to the results of the technique allowing As speciation, differences between measurements of the sum of species and target values are observed:

- in the previous table, particularly for samples containing (Q13, Q16) or enriched (Q15, Q18) with DMA
- in Fig. 2, with regard to the slope of the upper straight line in the graphs corresponding to the 2000 and 2001 samples.

This points to accuracy problems which are dealt with in the next paragraph.

3.6 Accuracy assessment

Commercial As standard solutions are available only in the case of inorganic species (mainly As_i^V). Methylated As standard solutions have to be prepared with Na salts of the corresponding acids (MMA and DMA). The old age of our laboratory reagents

was first considered as the cause of discrepancies between our results and target values given by CTQ.

A comparison on 6 urine samples was performed with the collaboration of Prof. X. Le (University of Alberta, Canada). Results are presented in Fig. 3: while they are highly correlated, results lead to slopes of regression lines which are not equal to 1.

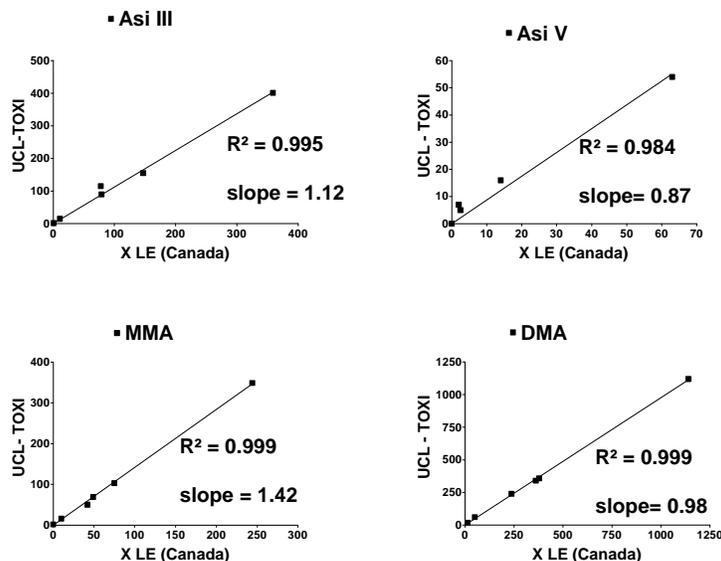


Fig. 3.

Comparison of results obtained for six urine samples by lab UCL-TOXI (y) and lab of Prof. X Le Canada (x). Uncertainty related to As standards induced a study which showed that some plastic tubes and vials can adsorb As species, particularly methylated ones.

A re-assessment of measurement accuracy involved the two following materials:

- BCR-627 Tuna Fish Tissue (Institute for Reference Materials and Measurements; Retieseweg, B-2440 Geel) containing $2.0 \pm 0.3 \mu\text{mol}$ dimethylarsinic acid/kg. The triplicate analyses of an aqueous extract of BCR tuna fish tissue powder gave the following results: 1.61, 1.78 and 1.83 μmol dimethylarsinic acid/kg, i.e. mean \pm SD : $1.73 \pm 0.11 \mu\text{mol}$ dimethylarsinic acid/kg (CV= 6.4%)
- CRM 18 Human Urine (National Institute for Environmental Studies, Ibaraki, Japan) containing $0.036 \pm 0.009 \text{ mg}$ As/L as dimethylarsinic acid. Triplicate analyses of CRM 18 Human urine were performed at one week interval during a month; triplicate means were 43, 41.9, 40.8, 45.8 and 43.8 μg As/L with CV between 1 and 5%.

Lastly, results of round robin # 29 and 30 organized by Prof. Dr. H. Drexler (on behalf of the German Society of Occupational Medicine and Environmental Medicine) are reported below:

Parameter	Sample	Our result	Reference value	Tolerance range
Σ As-U	A	38.9	37.5	28.1 – 46.9
	B	60.3	56.2	42.3 – 70.2
iAs ^{III} -U	A	13.6	12.6	9.2 – 15.9
	B	19.4	18.7	14.0 – 23.4
iAs ^V -U	A	4.1	4.7	2.8 – 6.7
	B	9.3	9.4	6.2 – 12.7
MMA-U	A	5.3	8.0	5.2 – 10.8
	B	9.7	12.9	8.9 – 16.9
DMA-U	A	12.8	15.8	10.0 – 21.5
	B	16.2	18.3	11.9 – 24.7
Σ As-U	A	20.4	20.7	15.2 – 26.2
	B	25.8	25.5	20.5 – 30.5

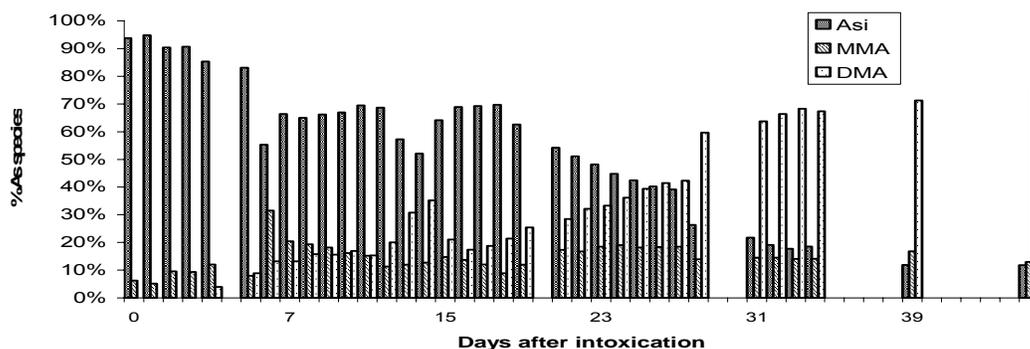
3.7 Technique validation

The usefulness of iAs metabolite urinary excretion as a biomarker of iAs absorption has been previously demonstrated (for a review see Lauwerys and Hoet, 2001b). Results of two additional studies (acute and sub-acute intoxication cases) using the presently developed technique confirm iAs biotransformations in the human organism.

- The urinary excretion of As metabolites was observed in a case of attempted suicide (ingestion of 9 g arsenic trioxide). All urine samples were analyzed using speciation. The profiles of the relative excretions of the As metabolites as a function of time are given in Fig. 4; observations are in agreement with other published data (Mahieu et al., 1981)

Fig. 4.

Percentages of As metabolites in urine as a function of time



- Asthma treatment may sometimes involve inhalation of arseniferous Spa water. Changes in the urinary excretion of As and its metabolites were assessed after a treatment period. In a group of 20 young boys mean urinary excretions were 0.3, 0.6, 0.3 and 9.5 before and 37.3, 27.9, 37.4 and 301.1 $\mu\text{g As/L}$ after the treatment for As_i^{III}, As_i^V, MMA and DMA, respectively. No need for statistics to assess As absorption during the treatment.

4. CONCLUSIONS

- High pressure anion exchange chromatography at pH 6.0 allows the specific measurement by AFS of As_i^{III}, As_i^V, MMA and DMA, the iAs metabolites in man; by comparison with previous techniques, speciation and full automation of analyses are improved; using 50 µl urine injections, lowest measurable concentrations of each species are around 1 µg As/L; measures can be replicated with CV (coefficient of variation) ≤ 10 and ≤ 5 % at the 2.5 and 10 µg As/L levels, respectively.
- Standard aqueous stock solutions of As species (1g/L) for instrument calibration must be prepared regularly (at least every two months; dilutions of working standards (up to 100 µg As/L) have to be prepared daily; tubes and vials have to be checked for possible adsorption of the analyte (in our hands, polypropylene is better than PVC or polystyrene).
- The sum of As species measured separately agrees with the amount of the directly reducible As forms assessed by an older technique using atomic absorption spectrometry.
- Urine samples can be kept refrigerated at 4°C during at least several weeks without changes of their As content; the valence state of iAs spiked in urine samples can change.
- With regard to accuracy, the agreement between observed and target values for DMA in two different “certified materials” appears satisfactory although results are at the lower and upper acceptable limits for the tuna fish extract and the human urine, respectively.
- Results obtained in 2002 for urine samples dispatched by the Centre de toxicologie du Québec in the frame of an interlaboratory comparison program indicate a slight overestimation (± 6-7%); this is also the case with samples from the Institute for Occupational, Social and Environmental Medicine in Erlangen, Germany.
- There is a need for MMA and DMA standard solutions for instrument calibration (presently only iAs^V standard can be found).

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PART II

DEVELOPMENT AND VALIDATION OF A METHOD FOR THE DETERMINATION OF N-TERMINAL STYRENE ADDUCTS TO HEMOGLOBIN

1. INTRODUCTION

Styrene is widely used in the production of plastics, resins and synthetic rubbers. High exposure through inhalation occurs in the production of fibreglass-reinforced styrene resins, which involve manual operations. The fate of styrene in humans with respect to uptake and disposition is well understood. The first step in the major metabolic pathway is the formation of styrene 7,8-oxide (SO) by the cytochrome P450-mediated monooxygenase system (CYP). SO is hydrated to styrene glycol by microsomal epoxide hydrolase (mEH) or conjugated with glutathione in enzyme-catalyzed reactions (glutathione S-transferases, GST). The major urinary excretion products, mandelic acid (MA), phenylglyoxylic and hippuric acid, are related to styrene glycol, indicating the intermediate formation of styrene oxide to be the major pathway of activation and detoxification of styrene, accounting for more than 85% of the absorbed dose. So, apparently there is less evidence for glutathione conjugation in humans. Saturation of metabolism occurs between 100 and 200 ppm styrene.

Styrene and its principal metabolite SO have been shown to be mutagenic and toxic to animals and humans. SO is able to form protein adducts (e.g. hemoglobin) and DNA adducts, which can be detected in humans. Therefore, the determination of SO adducts to N-terminal valine in hemoglobin as a measure of the biological effective dose. This parameter is chemical specific.

Styrene is classified by ACGIH as not classifiable as a human carcinogen (A4), by IARC as possibly carcinogenic to humans (2B). Styrene oxide is not classified by ACGIH, by IARC as probably carcinogenic to humans (2A).

2. METHODOLOGY

2.1 Required characteristics of the analytical method

To determine styrene adducts to hemoglobin in the N-terminal valine position, an analytical method had to be developed that possesses the following characteristics:

- specificity: SO adducts to valine must be distinguished from non-adducted valine and other amino acids, as well as from the other components of the complex matrix
- sensitivity: limits of detection must allow the determination of SO adduct in concentrations below 10 pg / 100 mg hemoglobin in human blood
- precision: the goal is to make measurements with a coefficient of variation < 10%
- accuracy: results of measurements in certified materials must lie in the acceptable range around the target value;

2.2. Reference materials

In order to test the analytical technique the following compounds had to be purchased or prepared by synthesis:

The primary standard is N-((RS)-2-hydroxy-2-phenyl ethyl)-Val-Leu-anilide (425.3 g/mol), at a certified purity by Bachem of > 98% by TLC and > 99% by HPLC.

Our laboratory as described under 2.3.5.2 prepares globin alkylated with styrene. This alkylated globin is calibrated against the primary standard and used as a secondary standard.

2.3 Analytical techniques

A modified version of the Edman degradation technique is used for the determination of N-terminal valine adducts to haemoglobin (Törnqvist et al., 1986; Severi et al., 1994). More specifically, the derivatization of globin is based on this technique: the N-terminal valine alkylated with styrene-7,8-oxide is specifically removed as a pentafluorophenyl thiohydantoin.

2.3.1 Chemicals

- Formamide (99+% spectrophotometric grade, Aldrich 29,587-6)
- Toluene (p.a., VEL 4118293)
- Pentafluorophenyl isothiocyanate (purum > 97% (GC), Fluka 76755)
- Ether (99.9% spectrophotometric grade, Aldrich 30,995-8)
- Pentane (99+% spectrophotometric grade, Aldrich 15,495-4)
- Toluene (99.8% HPLC grade, Aldrich 27,037-7)
- Hemoglobin, human, lyophilized powder (tested negative for HIV and Hepatitis B AG, Fluka H7379)
- d8-Styrene (Janssen, Beerse, Belgium)
- m-Chlorobenzoic acid (Janssen, Beerse Belgium)

2.3.2 Apparatus

- Agilent Series 6890N Gas Chromatograph coupled with:
- Agilent Series 5973 Mass Selective Detector (quadrupole mass spectrometer)
- Agilent Series 7683 Injector
- HP Vectra PC with Agilent Enhanced ChemStation software version G1701A

2.3.3 Preparation of blood samples

In freshly taken blood samples the erythrocytes are separated by centrifugation (10 min. at 3000 rpm). The erythrocytes are washed 3 times with isotonic saline and subsequently lysed by addition of 1 volume of distilled water. The cell membranes are precipitated by centrifugation at 5000 rpm during 1.5 h. This is followed by the addition of 25 ml HCl in 2-propanol (0.05M). This mixture is centrifuged (10 min. at 3000 rpm). Globin is precipitated from the supernatant with 40 ml ethyl acetate and isolated by filtration on a glass filter. The obtained globin is further washed with 15 ml ethyl acetate (2x) and 15 ml pentane (2x). Before storage at -20°C , globin is first dried in a dessicator.

2.3.4. Synthesis of pure globin

A quantity of 1 g human haemoglobin is dissolved in 7 ml isotonic saline. This procedure provides the same haemoglobin concentration as found in blood. The solution is stirred for several hours at 37°C to simulate the in vivo conditions. When the haemoglobin is completely dissolved, 50 ml of a 0.05 M solution of HCl in 2-propanol is added. The mixture is centrifuged during 10 min. at 3000 rpm whereby a reddish black precipitate is formed. The precipitate is discarded and 50 ml ethyl acetate is added to the supernatant to precipitate the globin (without the haem group). The further preparation steps as the same as for real blood samples.

This globin is used as blank reference material and to be added to standard solutions in order to reconstitute the same reaction matrix.

2.3.5 Internal standard preparation

To improve the reproducibility of the analytical method use is made of an internal standard. The use of a mass specific detection system allows for the optimal choice of a deuterated analogue as internal standard: d8-styrene oxide covalently bound to globin. Since neither the d8-styrene oxide nor its globin adduct are commercially available, both have to be prepared by synthesis.

If not otherwise specified, all chemicals and solvents used in these preparations are of analytical grade and used without further purification.

2.3.5.1 Preparation of d8-styrene oxide

d8-Styrene-7,8-oxide is prepared by adding m-chloroperbenzoic acid (1.73 g) in small portions over 10 min at 0°C to a stirred solution of d8-styrene (5 g) in dichloromethane (100 ml) and a phosphate buffer (100 ml, Na₂HPO₄ 0.1 M, NaH₂PO₄ 0.1 M, pH 8). The mixture is stirred for 5 h at room temperature, then cooled to 0°C, and again m-chloroperbenzoic acid (1.73 g) is added in small portions over 10 min. The mixture is stirred for another 5 h at room temperature. Then the organic phase is separated and washed with a saturated sodium thiosulphate solution and water and dried with disodium sulphate. The solvent is evaporated under vacuum and d8-styrene-7,8-oxide is purified with flash chromatography (silica gel-chloroform).

2.3.5.2. Preparation of globin alkylated with d8-styrene oxide

Haemoglobin (1 g) is dissolved in saline (7 ml), d8-styrene-7,8-oxide is added and the mixture is stirred for 60 h at 37°C. The alkylated globin (2-hydroxy-2-phenylethylvaline globin, d8-adduct) is then isolated and washed with ethyl acetate and pentane and dried by a gentle stream of air over the globin.

2.3.6 Derivatisation protocol

A 50 mg quantity of globin is dissolved in 1.5 ml formamide. To this 10 µl of the internal standard solution, containing 2.08 nmol of the d8-adduct and 1.2 mg globin per ml of formamide, is added. This mixture is made slightly alkaline by the addition of 30 µl of a 1M NaOH solution. Finally, 15 µl pentafluorophenyl isothiocyanate is added as derivatizing agent. The reaction mixture is stirred overnight at room temperature and a further 1.5 h at 45°C until completion of the reaction. Afterwards, the mixture is extracted with 2 ml ether (3x). This is done under gentle shaking to avoid the possible formation of an emulsion. The combined ether fractions are evaporated to dryness under a stream of nitrogen. The residue is redissolved 1 ml toluene. To remove the

remaining formamide this solution is washed with 2 ml water and subsequently shaken with 2 ml NaHCO₃ (0.1 M) to hydrolyse possible by-products. After a final washing with 1 ml water, toluene is evaporated under a stream of nitrogen. The residue is redissolved in 40 µl toluene and frozen for 1 h at -18°C. After removal of the precipitate which is formed, the samples are ready for gas chromatographic / mass spectrographic analysis.

2.3.7 GC/MS procedure

The chromatographic separation of the different components in the samples is made on a DB5-MS fused silica capillary column (30m x 0.25mm, 0.10 µm phase thickness, J&W 122-5531). Helium is used as the carrier gas. The gas chromatograph is operated in the splitless mode under a constant flow of 2.5 ml/min. The injector is kept at 230°C. The initial oven temperature is 90°C for 0.1 min after injection, followed by a linear temperature program up to 280°C at a rate of 20°C/min. With the aid of an automatic sampler 5 µl aliquots of the samples are injected. Between samples 2 blank runs are made with a solution of 0.01% polyethylene glycol in toluene to purge the instrument from remaining impurities.

The temperature of the interface between the gas chromatograph and the mass spectrometer is set at 280°C and the source pressure at 0.005 Pa. The instrument is operated in the electron impact mode with an electron energy of 70 eV. Analysis are carried out in the single ion monitoring mode. The covalent binding of the styrene-7,8-to valine results in the formation of two diastereoisomers. The two isomers are chromatographically separated. The identification of the peaks is assured by the determination of the retention time relative to the internal standard. Both isomers show a base peak in the mass spectrum at 325 amu due to loss of a (C₆H₅)CHOCH fragment, accompanied by proton shifts. The original valine nitrogen in the positive daughter ion carries away one hydrogen atom of styrene. The d8-adduct, which is used as internal standard, shows a corresponding base peak at 326 amu. Because of their high abundance (sensitivity) and high mass number (specificity), these ions are used for the selected ion monitoring analysis.

2.3.8 Calibration

Calibration curves are prepared through the addition of different amounts of the reference alkylated tripeptide to a constant amount of the d8-internal standard.

2.4 External validation

A field study is conducted to investigate the quantitative relationship between styrene exposure and haemoglobin adducts in workers. This phase in the project is called the external validation. The aim of this part of the project is to establish the usefulness of the technique of adduct determination in the process of risk estimation of exposure to known mutagens/carcinogens in human populations.

2.4.1 Study groups

The field study is conducted in co-operation with our partners from the VUB and UCL. The subjects of this study are a group of 44 workers from the same factory with exposure to styrene and a matched control group.

2.4.2 Sampling for biomonitoring

Biomonitoring is done in two separate periods. First, between 7/9/2000 and 7/12/2000 urine samples were collected for the determination of mandelic acid (MA) as biomarker of styrene exposure. A period of minimally 3 months is chosen because of the relatively long life of haemoglobin (approx. 3 mo). With exposure on each working day it is expected that adducts will accumulate over such a time period. Therefore, an equally long monitoring period is necessary to correlate possible blood adducts to the exposure. During that period urine samples are collected from each participating worker on every Thursday (end of shift). Urine samples are kept frozen (-24°C) prior to the analysis.

Secondly, starting on 7/12/2000 and ending on 8/2/2001 blood samples are collected in groups of 8, each Thursday except during Christmas holiday. Fresh blood samples were processed as outlined in section 2.3.3 to isolate globin. Globin is stored frozen at -70°C until the time for analysis.

In March 2001 two companies are selected in the same geographic region to provide a control group. For each exposed worker two control persons are chosen, matched for age, smoking and drinking habits, socio-economic status. Sample collection started on 27/04/2001 and ended on 08/06/01.

2.4.3 Exposure assessment

To assess the average individual worker exposure during the three months preceding the blood sampling for adduct determination, urine samples are collected from each subject for the measurement of mandelic acid (MA), the main styrene metabolite.

2.4.3.1 Determination of Mandelic Acid

To 1 ml aliquots of urine are added 0.1 ml of HCl_{conc} and 0.1 ml of an internal standard solution containing 0.6 mg/ml 4-hydroxybenzoic acid in methanol. The mixture is extracted with 3 ml ethyl acetate. 1 ml of the organic layer is evaporated to dryness under a stream of nitrogen and redissolved in 1 ml of H₂O_{dist} to form the final solution for HPLC analysis.

Separation and quantification is done with the aid of a Varian Series 9050 Liquid Chromatograph in reversed phase mode (Nucleosil₁₂₀₋₇ C₁₈, 20 cm) and UV detection at a wavelength of 220 nm. The mobile phase is an isocratic mixture of 20 % methanol and 80 % of a 0.5 % solution of acetic acid in H₂O_{bidist}. The flow rate is set at 1.5 ml/min and the column temperature at 35°C. Injections are made with an autosampler equipped with an injection loop of 25µl.

2.4.3.2 Calculations

Exposure to styrene (in ppm) is calculated from MA concentrations corrected for creatinin excretion (MA_{cr}). The calculation is based on the following empirical regression equation:

$$C_{\text{exp}} \text{ (in ppm)} = 65 \times \text{MA}_{\text{cr}} \text{ (in mg/g)} - 3.6$$

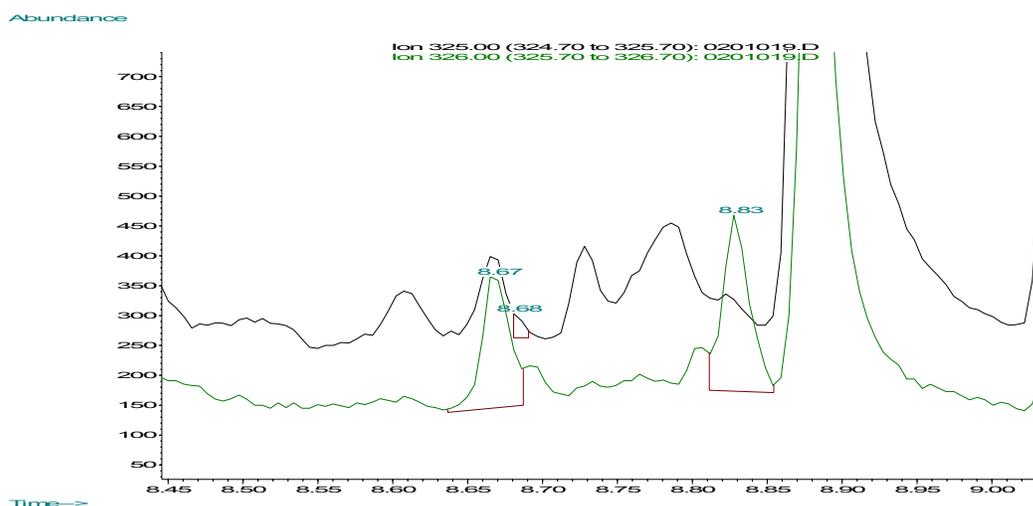
The detection limit of the MA assay is 50 mg/l urine. In each case were MA is below the limit of detection, a default of 25 mg/l is assumed in the expression above.

3 RESULTS

3.1 Internal validation

3.1.1 Minimising background noise

The first tests of the analytical method for valine adducts with blank globin samples revealed a considerable background noise (fig. 1). Investigating and minimising the causes of this noise is a prerequisite in the early stages of method development, since many aspects of the validation process are dependent on it. The noise level critically influences especially the limit of detection, the specificity, the accuracy and the precision.



**Fig. 1 Chromatogram with background noise
(the abundance of ion 325 is compared to ion 326 of the internal standard)**

The first efforts to ameliorate this problem are concentrated on removing possible contaminants that can be present in the sample or the in reactants used. Therefore, a more thorough sample clean-up procedure is tried out to eliminate contaminants from the matrix. This procedure consisted of an extra purification step through redissolving the dried residue in 60% methanol-water and extracting with 2 ml pentane. After evaporation to dryness of the pentane fraction, the final residue is redissolved in 40 μ l toluene as in the original protocol. Tests are done to compare this revised protocol with the original. The results do not show any obvious improvement of the noise levels in the chromatograms. Therefore, the extra purification step is not added to the protocol.

The second step consists of a series of trials by elimination in order to identify possible sources of external contamination during the processing of the blood samples. It is discovered that the neoprene septa, used to tightly close the reaction vials, are probably an important source of impurities. A solution to avoid transfer of impurities to the sample is to use Teflon septa. An additional improvement of the background noise level is obtained through a more rigorous procedure for cleaning and rinsing the reaction after use.

Fig.2 shows the results of these changes to the analytical procedure.

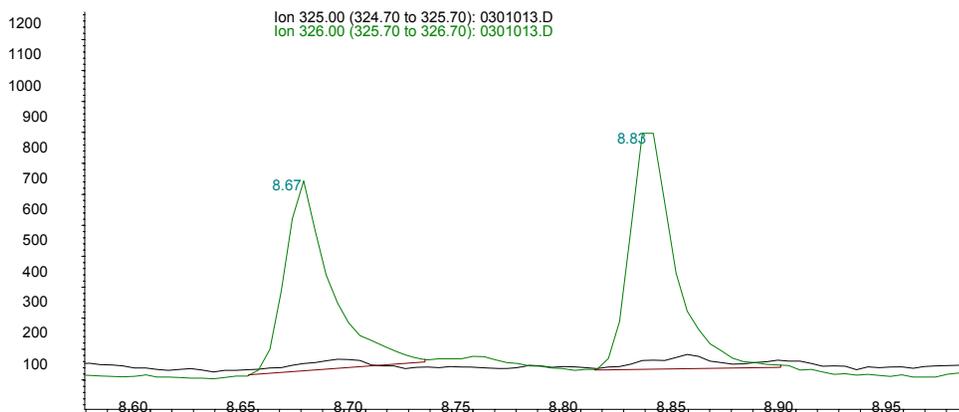


Fig.2 Example chromatogram with reduced background noise

3.1.2 Contamination with SO-adduct

The calibration procedure for each batch of analytical samples also includes the use of negative controls. Blank samples consist of 50 mg globin prepared as described in section 2.3.4 from human haemoglobin purchased from Sigma. In the early stages of the method development and validation, the blank samples show chromatographic peaks at the same retention times as the styrene-7,8-adduct isomers. To investigate the possible source of this contamination, globin from Sigma compared with globin obtained from volunteers at our lab. Also samples containing no globin are used in this investigation. The prime result shows variable levels of contamination in nearly all samples (fig. 3).

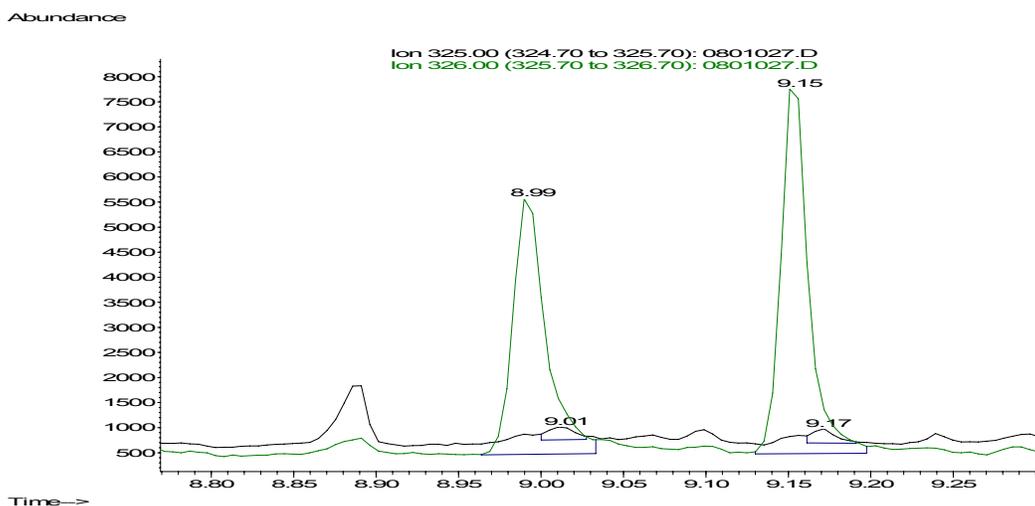


Fig. 3a Chromatogram with low level of contamination (compared with the internal standard)

Even samples of the same globin can show highly variable amounts of contamination with SO-adduct. An important observation is the fact that the results are poorly reproducible within the same batch of globin or between batches of globin obtained from the same person. Even different globin samples from the same person prepared

on the same day with the same materials from freshly obtained blood show considerably divergent results. This observation indicates that the source of the contamination is not the blood (or haemoglobin) itself or the preparation procedure of the globin. In that case more reproducible results are expected within each batch of globin.

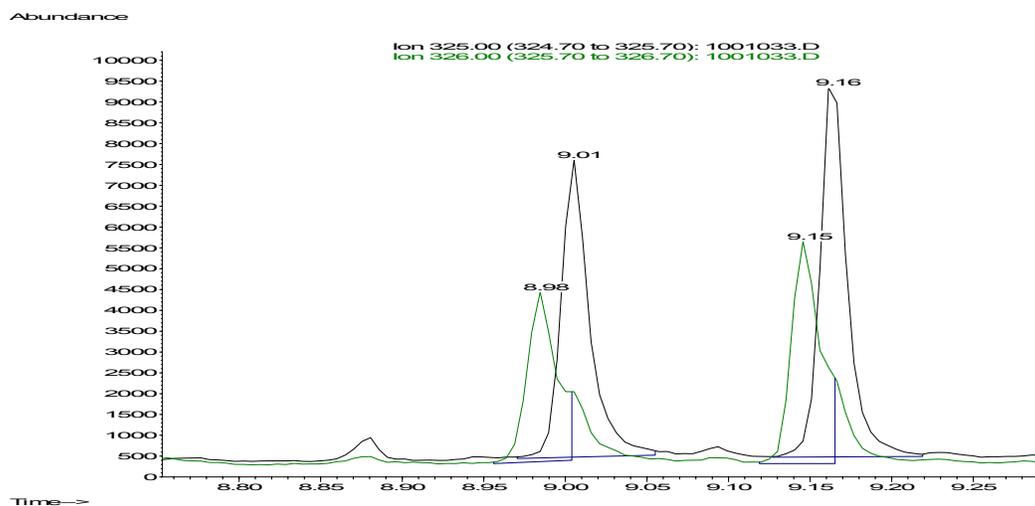


Fig. 3b: Chromatogram with high level of contamination (compared with the internal standard)

It is most likely that the source of the contamination stems from the materials and/or reactants used in the derivatisation procedure of the globin samples. To test this hypothesis all reactants, glassware and other materials had to be replaced. The results indicate that contamination of laboratory glassware is the most important contamination source. Therefore, in the analytical procedure all containers for globin during the derivatisation procedure are to be discarded after single use. For non-discardable glasswork a more thorough cleaning procedure is adapted consisting of the following steps: rinsing with acetone, soaking overnight in mucasol, multiple rinsings with distilled water until foam-free, final rinsing with acetone and drying.

3.1.3 Calibration curve

Fig. 4 shows a typical example of a calibration curve with 5 adduct concentrations and a blank. The data are summarised in table 1.

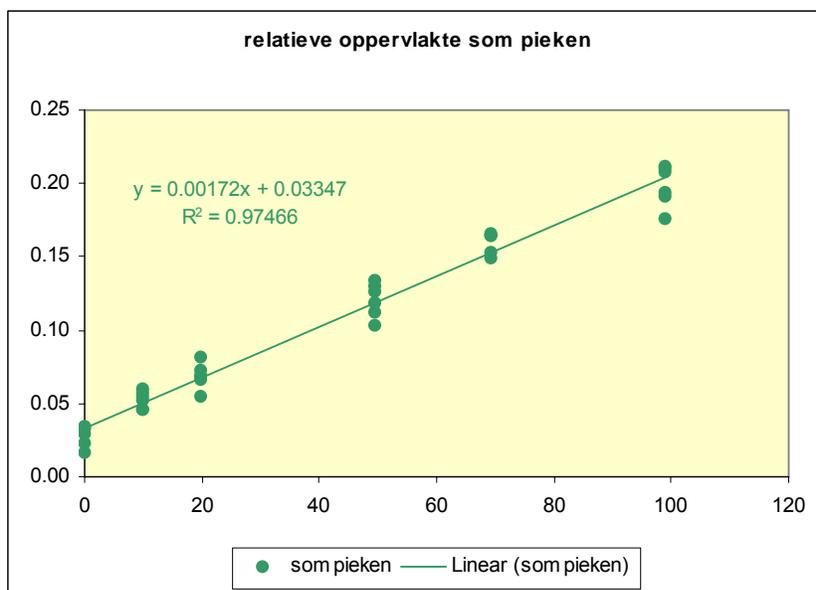


Fig.4: Calibration Curve

Concentration (pmol/g haemoglobin)	Average Peak Ratio* (N=6)	Coefficient of Variation (%)
0	0.028	25.5
9.9	0.053	11.4
19.8	0.069	12.1
49.4	0.121	9.5
69.1	0.159	4.9
98.8	0.198	7.1

(*) Ratio between the sum of the peak areas for the 2 adduct diastereoisomers (amu = 325) and the sum of the peak areas for the internal standard (d8-adduct, amu = 326)

Table 1: Summary of data for the calibration curve

3.1.4 Precision

Estimation of the precision is based on replicate analysis of standard solutions at 3 different concentrations and blank samples (Table 2). For each concentration level 3 samples are usually prepared and analysed within a single day. This procedure is repeated on 7 different days. The variability as measured by the coefficient of variation is thus an aggregate of both the within day and between days variability, reflecting the overall precision of the method. Taking into account the extremely low concentration levels that are to be measured, a coefficient of variation of 19.8% for blank samples and between 10.9 12.1 % for the standard solutions is acceptable.

Adduct conc (pmol/g hb)	Number of samples	Mean Peak Ratio (amu 325/amu 326)	Coefficient of Variation
0	25	0.056	19.8 %
4.9	18	0.067	11.0 %
19.8	16	0.095	12.1 %
39.5	18	0.137	10.9 %

Table 2: Precision of replicate analysis

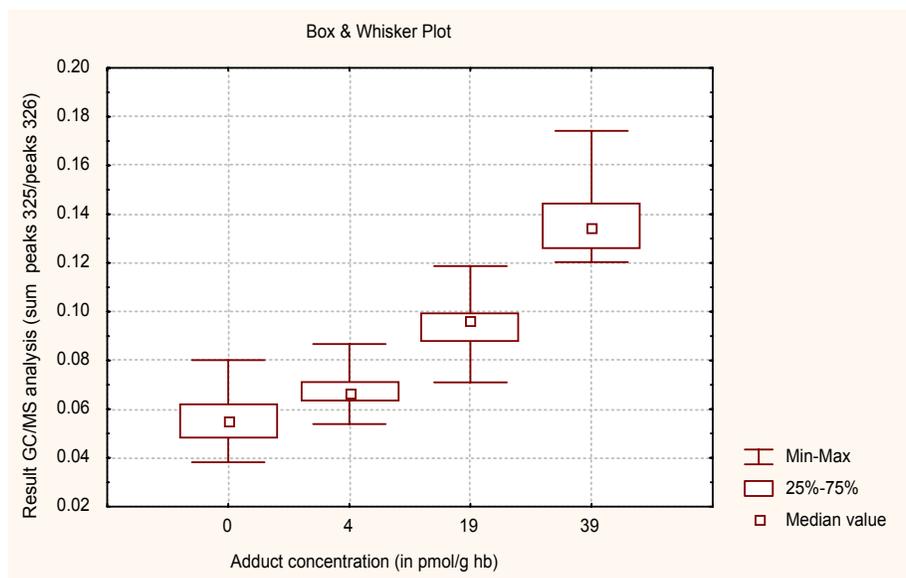


Fig. 5: Plot of the analytical precision data sets

3.2 External validation

3.2.1 Study groups

The exposed and control groups both consisted of 44 male employees with average ages of respectively 41.4 y and 40.9 y (table 3)

	Exposed	Controls
Number	44	44
Age (Mean +/- SD) (Range)	41.4 +/- 9.3 21 - 59	40.9 +/- 8.9 22 - 58
Alcohol Consumption (drinks/week)	8.7 +/- 9.3 0 - 35	9.9 +/- 10.5 0 - 39
Smokers (N)	20	20

Table 3: Descriptive parameters of the study groups

3.2.2 Styrene exposure of the exposed group

In the group of resin laminators urine samples are taken every week for a period of 3 months. This gives minimum 11 and maximum 20 daily exposure estimates per worker by monitoring the urinary mandelic acid (MA) excretion. Table 4 summarises the average urinary MA excretions in the exposed group and the calculated styrene exposures.

	N	Mean (+/- SD)	Minimum	Maximum
MA (mg/g creatinine)	689	201.6 (+/- 148.3)	40.6	618.2
Styrene (ppm)	689	9.5 (+/- 9.6)	neg	36.6z

Table 4: Measured MA excretions and estimated styrene exposures

The average calculated styrene exposure amounts to 9.5 ppm for an 8h working day. The highest exposed worker has an estimated styrene exposure of 36.6 ppm. Only 8 out of 44 workers have an average exposure above 20 ppm. Fig. 6 depicts the observed urinary MA and styrene exposure distributions.

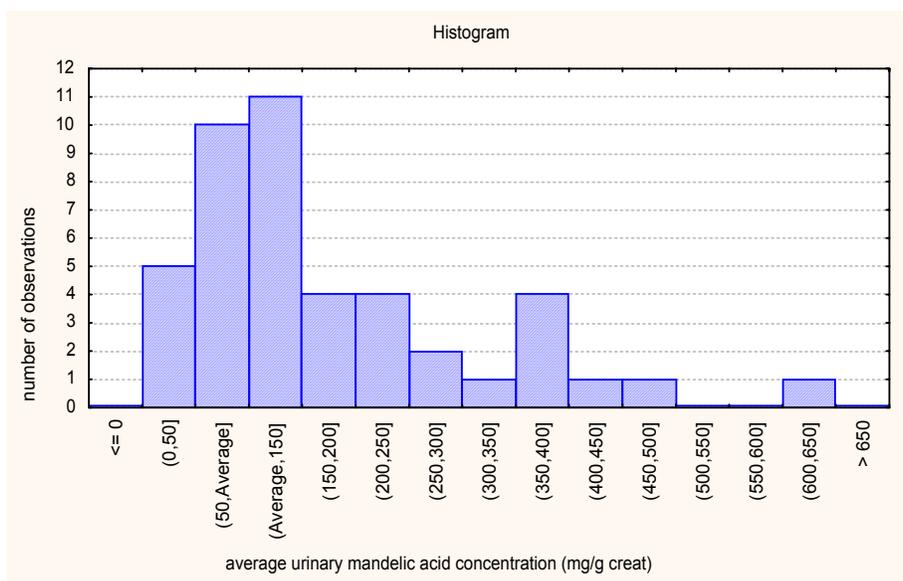


Fig. 6a: Distribution of individual average urinary MA concentrations

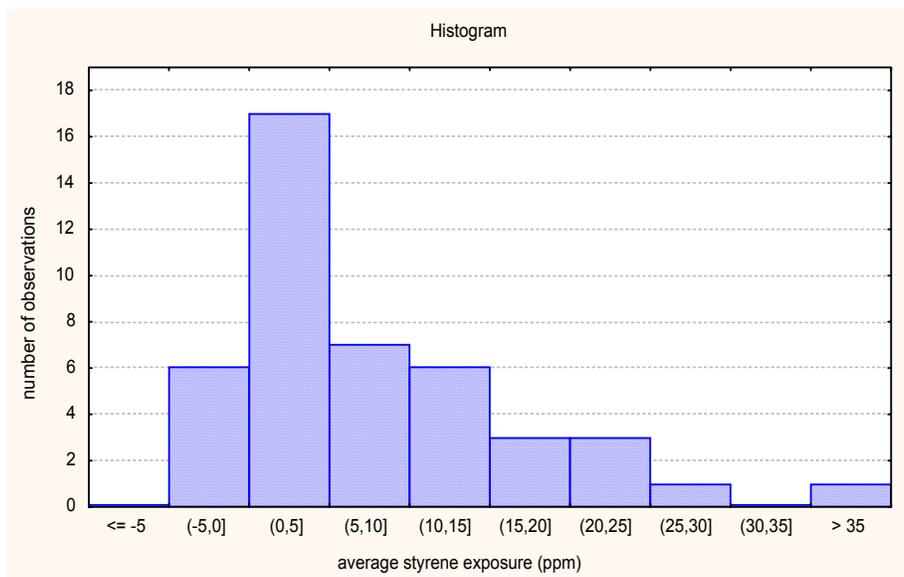


Fig. 6b: Distribution of individual average styrene exposures

3.2.3 Expected adduct levels

The external validation of the adduct measurements also entails a comparison with the available data from literature. The basis for this comparison is a by Christakopoulos et al, 1993. This study determined haemoglobin adduct concentrations in workers exposed to styrene (mean 1931 ppmh/week, range: neg-7151). The data that allow a quantitative comparison with our results are summarised in fig.7.

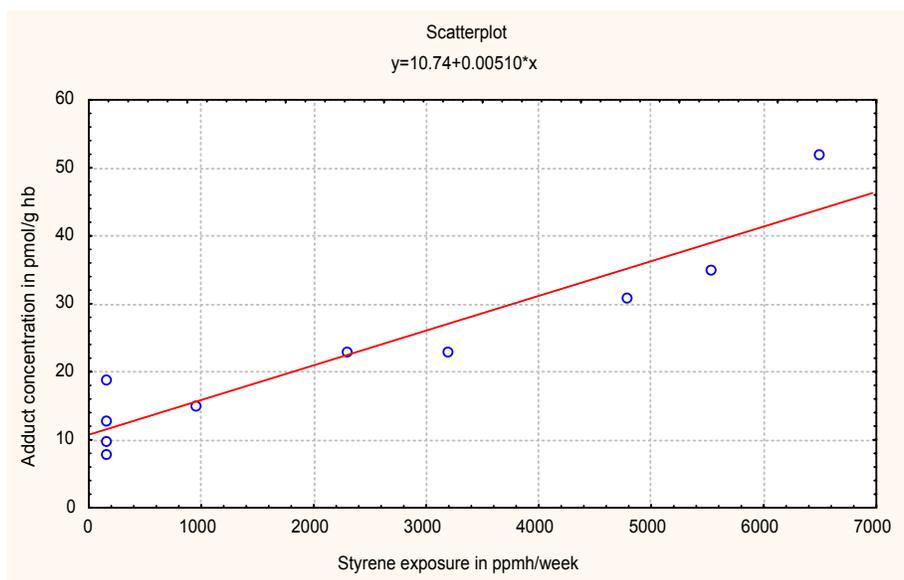


Fig. 7: Styrene exposure and haemoglobin adduct levels

To determine the expected adduct level at the exposure levels in our study we use the following regression equation derived from that data set:

$$y = 10.74 + 0.00510x.$$

This means that with a mean exposure of 269.15 ppmh/week in the current study we expect a mean haemoglobin adduct concentration of 12 pmol/g hb (maximum 17 pmol/g hb).

It has to be observed that there are two reasons why the agreement between this expected value and our own result can not be expected to be very precise. First, there is the difference between styrene exposures in the two studies. In order to calculate the expected value for our study, we had to use the lowest end of the regression equation from the Christakopoulos study. The result is not much higher than the intercept of the regression equation suggesting a relative high uncertainty. Second, all data points from the Christakopoulos below the (unknown) limit of detection are omitted from the regression calculation. This will tend to overestimate the values of the slope and intercept, rendering the predicted value to high.

3.2.4 Measured adduct levels

The analysis of the blood samples of our field study is still in progress. The data that can be preliminarily be reported here are from the first 24 exposed workers and their matched controls. The average adduct levels for the two groups are respectively 6.85 pmol adduct / g haemoglobin (sd: 2.85) and 2.12 pmol adduct / g haemoglobin (sd: 1.75). The difference is highly statistically significant ($t = 6.85$, $p = 0.01$).

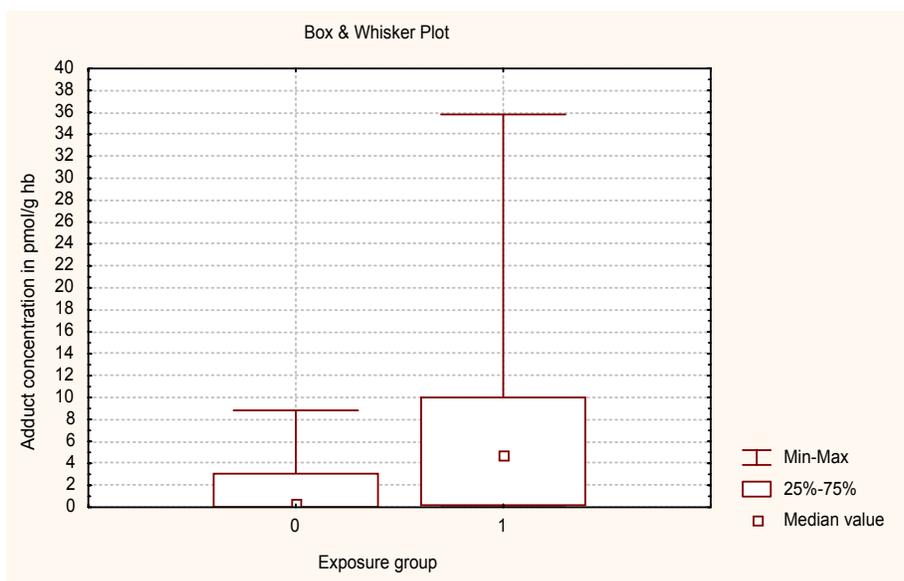


Fig. 8: Adduct levels in unexposed controls (0) and in exposed workers (1)

The mean adduct value is in reasonable agreement with the expected value derived from the data of the Christakopoulos study. The possible explanations for the difference are explained in the previous paragraph.

4. DISTRIBUTION AND VALORISATION

The results and experiences of the present will be shared and valorised in the following ways:

- Through publication of the relevant data in the scientific literature
- By disseminating information on the practical possibilities of the technique in forums for the postgraduate education of occupational physicians and hygienists. Also workers in the field of environmental health may be interested in the use of these biomarkers.
- By offering laboratory facilities and support to field workers interested in the application of these new tools.
- By sharing information and collaborating with other research institutions willing to accomplish work in this field

5 PERSPECTIVES

It may be anticipated that the results of this work can lead to further applications and research. For the immediate future, the following possibilities may be envisaged:

- Adaptation of the technique to cover related mutagens, especially epoxides or epoxide forming compounds such as ethylene and ethylene oxide, propylene and propylene oxide.
- Adaptation of the technique for other electrophilic mutagens
- Development of protocols for using adduct levels in the assessment of cancer risks.

ACKNOWLEDGEMENTS

We thank Belgian Science Policy for its support that made these investigations possible. Also we like to thank the members of the Committee for the Follow-Up for contributing many constructive comments that helped to improve the quality of the study. Special thanks to the management and employees of the enterprises for allowing and participating in our field study. Finally, we have to thank our colleagues in this network for their fruitful collaboration in this endeavour.

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PART III

DEVELOPMENT AND VALIDATION OF THE MICRONUCLEUS TEST AND THE COMET ASSAY

1. INTRODUCTION

1.1 Micronucleus test

Micronuclei arise from chromosomal material that is left behind during anaphase and did not migrate correctly to 1 of the 2 poles, to be included in the main nucleus of one of the daughter nuclei. Micronuclei can contain acentric fragments, but also whole chromosomes (Adler, 1984).

Three mechanisms are recognized for the development of micronuclei and micronucleus like structures (Heddle *et al.*, 1991): (1) mitotic loss of acentric fragments: mechanical consequences of chromosome breaks and exchanges, (2) mitotic loss of whole chromosomes, and (3) apoptosis.

Not all single strand breaks give rise to the formation of micronuclei in the following cell division, and some will do so only in the second or third consecutive division. For the formation of micronuclei giving indication of cytogenetic damage, cell division is required.

The micronucleus test was developed essentially to detect structural chromosome damage induced by chemicals (Matter en Schmid, 1971; von Ledebur en Schmid, 1973; Heddle, 1973; Matter en Grauwiler, 1974; en Schmid, 1975).

In 1985 Fenech and Morley developed a *Cytokinesis-Blocked* (CB) method that allowed to *in vitro* distinguish cells that have divided once and cells which did not divide or divided more than once. In combination with the classical micronucleus test (in interphase) we now have the CBMN-test which is easy to perform, fast and reproducible and moreover giving a lot of information: not only a discrimination between micronuclei in cells that have divided and micronuclei in cells that haven't divided, but also a very good estimation of the mitotic delay of the cell cycle (which is a measure for general toxicity).

The test was thus initially developed to estimate the induction of chromosomal damage resulting from the action of clastogens, but also model aneugens like colchicine and vinblastin were able to induce micronucleus formation. Hence, the test also seemed to be useful for the detection of aneuploidy. An extra step was needed to allow the differentiation between clastogen induced micronuclei and aneugen induced micronuclei.

In 1980 Yamatoto and Kikuchi distinguished these micronuclei by measuring the diameter of the micronuclei (Yamatoto and Kikuchi, 1980). Later Vig and Swearngin (1986) demonstrated that the kinetochores in the micronuclei could be visualized with CREST serum (CREST is short for calsinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactuly, and telangiectasia, patients suffering from this disease produce antibodies against their own kinetochores, and these antibodies are species specific) (Vig en Swearngin, 1986). Yet other ways to discriminate these clastogen and aneugen induced micronuclei were demonstrated in our laboratory (Vanderkerken *et al.*, 1989), namely by using the '*Fluorescence In Situ Hybridization*' or FISH technique with centromeric probes leading to centromere positive (aneugen

induced) and centromere negative (clastogen induced) micronuclei (C⁻-MN and C⁺-MN), or by measuring the surface and the DNA content of the micronuclei.

In 1997 Kirsch-Volders *et al.* described the *in vitro* micronucleus test as a multi-endpoint assay to simultaneously detect mitotic delay, apoptosis, chromosome breakage, loss and non-disjunction. They mention that frequency of apoptotic figures in both mononucleated and binucleated cells provides a measure for cell death before or after cell division. In 2001 Kirsch-Volders and Fenech again mention that including necrosis and apoptosis information provides a more comprehensive cytokinesis block micronucleus assay for biomonitoring.

1.1.1. Biomonitoring: Use of more specific reporter cells

In human biomonitoring studies the cells most commonly used are peripheral blood lymphocytes in the *ex vivo/in vitro* cytokinesis blocked micronucleus test (CBMN test) or in the regular *in vivo* micronucleus test, for the simple reason that peripheral blood lymphocytes are easy to obtain and to cultivate. Very often, these peripheral blood lymphocytes are actually not the target cells of certain chemicals, nor the cells being exposed to them directly. Cells of the gastro-intestinal tract for instance are the cells exposed directly when exposure through ingestion is considered, or nasal and buccal mucosa cells or long epithelial cells in sputum (Emmery *et al.*, 2001) when exposure through inhalation is considered. This exposure through inhalation is relevant in the case of exposure to chemicals like chromium, cobalt and styrene, and for this reason, the laboratory tried to use these cells in both the Comet assay and the micronucleus test (without further cultivation of the cells *in vitro*).

1.1.2. Biomonitoring: *ex vivo/in vitro* Micronucleus test in mononucleated and binucleated cells

Human biomonitoring of early genetic effects demands accurate, sensitive, and if possible not too time consuming methods to estimate the presence of mutations. One of the most promising techniques until now is the cytokinesis blocked micronucleus test which can detect both chromosome mutations and genome mutations in binucleated cells. Plenty of literature has been published on this method, but before strongly recommending this method it is essential to evaluate both its' negative as positive features. Fenech *et al.* (1999) summarized the advantages of the test. Nevertheless, until now, nobody took the presence of micronuclei in mononucleated cells into account, although it can be very complementary to the information embodied in binucleated cells. In 2001 Kirsch-Volders and Fenech suggested to integrate the frequency of micronuclei in mononucleated cells in the CBMN test in biomonitoring studies, of which Micheline Kirsch-Volders is one of the 5 coordinators (Kirsch-Volders and Fenech, 2001).

A large intra- and inter-laboratory (20 laboratories) variation study was performed in an international slide-scoring exercise by the HUMN project (Fenech *et al.*, 2002).

1.1.3. Towards validation of the *in vitro* micronucleus test

Quantification of micronuclei in cells treated *in vitro* is considered as a very useful new genotoxicity test because of its' simplicity, the possibility of automation using image analysis, its' power to detect both clastogens and aneugens, and on top of all this, the possibility to obtain mechanistic data (chromosome non-disjunction versus chromosome loss and chromosome breaks) (for review, see Kirsch-Volders *et al.*,

1998). For this reason it is not surprising that the International Workshop on Genotoxicity Testing (IWGT) selected the *in vitro* micronucleus test as one of the four new tests qualified for discussion in Washington in 1999.

Considering the work schedule and the possible results of the *in vitro* micronucleus test, the 1999 work group focused their discussion on the first step, namely the detection of micronuclei. The fact that the micronucleus test can be used to obtain mechanistic information was acknowledged by the work group, but not taken into consideration in their discussions. The main goal of this workgroup was to recommend a protocol for a reliable *in vitro* micronucleus test for the detection of both clastogens and aneugens. Practically, emphasis was placed on the design of a protocol that should put strength on negative results and that should avoid false positive results (Kirsch-Volders *et al.*, 2000).

The recommendations of the work group are given in the section 2.2.3..

Because a number of important *in vitro* micronucleus validation studies were in progress at the time of the 1999 Washington IWGT, it was not possible to design a definitive, internationally harmonized protocol at that time. These studies have now been completed and the data were reviewed at the '3rd International Workshop on Genotoxicity Testing' (Plymouth, June 2002). Data from studies coordinated by the French Society of Genetic Toxicology, Japanese collaborative studies, European pharmaceutical industry validation studies, along with data from Lilly Research Laboratories were used to prepare conclusions on the main aspects of the *in vitro* micronucleus protocol. The consensus agreements on the protocol for performing the *in vitro* micronucleus are given in section 2.2.3.(Kirsch-Volders *et al.*, 2003).

1.2 Comet assay

The Comet assay is a micro gel electrophoretic technique that allows the measurement of DNA damage in a cell by cell approach. The alkaline version (the most sensitive and most commonly used version) of this technique, detects DNA breaks, alkali labile sites, open repair sites and cross linking (Singh *et al.*, 1988). This technique is used as an *in vitro* and an *in vivo* genotoxicity test on different cell types of different species and also in biomonitoring studies and to study DNA repair mechanisms (reviewed in Anderson *et al.*, 1998; Collins *et al.*, 1997; and Tice, 1995). Cells are embedded in agarose and placed on a microscopic slide and then lysed in a solution rich in detergents and salts. After denaturation of the DNA in an alkali buffer the electrophoresis is performed leading to the migration of DNA fragments towards the positive pole of the electrophoresis unit. The quantity of DNA migrating is dependent upon the size of the DNA molecule and upon the number of broken DNA ends. After neutralization of the pH and fluorescent staining of the DNA, comet like figures can be detected under the microscope. With an image analysis system coupled to specialized software (Komet 5.0, Kinetic Imaging, Ltd., Liverpool U.K.) different damage parameters can be measured. The most commonly used parameters are '*tail length*', measured from the centre of the head of the comet to the end of the tail, '*tail DNA*', the percentage of DNA in the tail based on the fluorescence intensity, and '*tail moment*', the product of the 2 previous parameters.

Besides its' sensitivity, the Comet assay is easy to perform and fast, and doesn't require a very large financial investment. A drawback of the technique is that the types of damage detected with the technique can be repaired fast and easily and by consequence the damage is measurable during only a short period.

The Comet assay is considered to be a good indicator of exposure and to a lesser extent of biological effect. Recently at the '*International Workshop on Genotoxicity*

Testing, organized by ICH ('*International Conference on Harmonization*', March 1999, Washington DC) guidelines for performing the alkaline comet assay were proposed. This means that this fairly recent technique, together with already existing testing batteries, qualifies for insertion in the legislation for the testing of new chemicals.

1.2.1. Internal standard

The electrophoresis step is the most critical step of the method and is very sensitive to experimental variation. This can be minimized by the systematic use of an internal standard in every electrophoresis run.

An internal standard to be used in the Comet assay was developed in the laboratory (De Boeck *et al.*, 2000).

1.2.2. Use of more specific reporter cells

see 1.1.1..

1.2.3. Use of the Comet assay in the Global Repair Phenotype assay

The Comet assay can be used for the *in vitro* evaluation of individual repair capacity. The rationale to this suggestion is that following the DNA repair with time (15', 30', 60', 12h and 24h) using the alkaline version of the Comet assay could give a good estimation of the DNA repair capacity. This repair capacity is influenced by the genotype of the individual, but also by possible previous exposure to a mutagen. One can suggest that previous for instance chronic exposure to a mutagen could activate the synthesis of DNA repair enzymes, leading to a faster and/or better repair of the DNA damage induced by this compound (adaptive response). One can also imagine that a chronic exposure induces genetic changes leading to a lower repair capacity (cumulative response). To test this, the laboratory developed the Global Repair Phenotype assay (Touil *et al.*, 2002)

1.2.4. Influence of folic acid on DNA damage and repair

Folic acid is essential for the synthesis and repair of DNA. In epidemiological studies, dietary folic acid deficiency is associated with an increased risk of several specific malignancies, notably cancer of the cervix, lung, large intestine and brain (Duthie and Hawdon, 1998). Two primary mechanisms are believed to link folic acid levels to the development of these cancers, alteration of gene expression and increased DNA damage (Choi and Mason, 2000). Earlier, Branda *et al.* (1991) found that low serum folic acid levels were associated with increased mutation frequency of the *hprt* locus in women undergoing chemotherapy. In addition, Fenech and Rinaldi (1994) observed that folic acid deficiency promotes instability in human DNA, causing damage as increased micronuclei in erythrocytes and lymphocytes. Waifan and Poirier (1992) found that there was an increase in activation of the oncogenes *c-myc* and *c-fos* in rats fed a methyl-deficient diet. Kim *et al.* (1997) showed that folic acid deficiency in rats could produce hypomethylation specifically within the *p53* tumor suppressor gene potentially causing activation of the gene's suppressing properties. Other studies (Duthie and Hawdon, 1998; Duthie, 1999; Duthie *et al.*, 2000) have demonstrated a link between folic acid deficiency and DNA instability, including strand breakage, reduced DNA synthesis and repair and uracil misincorporation.

The general aim of this part of the project was to determine the effect of folic acid, vitamin B6 and vitamin B12 deficiency on DNA damage and repair at concentrations that are in a physiological range. We hypothesized that under these conditions the global repair phenotype assay will be more useful in discriminating between those

individuals who repair well and those individuals who repair poorly and therefore become more susceptible to the effects of DNA damaging agents. We focused on the influence that increasing folic acid concentrations might have on DNA damage and repair. Parallel studies were conducted within the Belgian Science Policy project on susceptibility and occupational exposure to styrene. Therefore the corresponding active mutagen styrene oxide was used to challenge the cells.

2. METHODOLOGY

2.1. Micronucleus test

2.1.1. Biomonitoring: Use of more specific reporter cells

Nasal cells are obtained by brushing a cell brush (Medscand Medical, Accellon Multi, Biosampler) along the nostrils. The cells are then suspended in 2ml of physiological salt, and then cytopun (Cytospin, Shandon, Analis) onto microscopic slides. The samples can be analyzed by fluorescence microscopy (Axioskop, Zeiss, using a UV filter, excitation 490 or 365nm) after acridine orange staining. Presence of mucus on the slides leads to a non-uniform staining, which we tried to improve by treating the slides with Otrivine® or pepsin, but which didn't help.

The most important features of using these cells is that they are the direct contact cells of styrene for instance, and that the method to obtain these cells is not invasive; unfortunately, the staining is not optimal yet.

2.1.2. Biomonitoring: the ex vivo/in vitro micronucleus test in mononucleated and binucleated cells

Mononucleated cells will show damage already present *in vivo* before the start of the cultures, while binucleated cells can contain micronuclei that either were already present at the start of the cultures or arose from lesions during division in culture. The conclusion was that a more sensitive estimate of DNA damage and genome mutations can be achieved when the following endpoints measurable in the CBMN test are taken into account: (1) micronuclei in mononucleated cells; (2) micronuclei in binucleated cells; (3) apoptotic cells; and (4) necrotic cells.

Twenty four hours after PHA-stimulation is probably the optimal sampling time to assess the presence of micronuclei in mononucleated cells, and the presence of apoptotic and necrotic cells. Gathering the same information at the same time in the binucleated cells is at the same time practical and can give extra information (Kirsch-Volders and Fenech, 2000).

2.1.3. Towards validation of the in vitro micronucleus test

The *in vitro* micronucleus test is unique in the sense that it allows parallel estimation of mitotic delay (frequencies of binucleated cells), apoptosis (frequencies of condensed nuclei), chromosome breakage/chromosome loss (frequencies of micronuclei with or without centromere/kinetochore labeling) and chromosome non-disjunction (distribution of double-labeling in macronuclei of binucleated cells). The following scheme gives the *rationale* and possibilities of the test as developed by our laboratory.

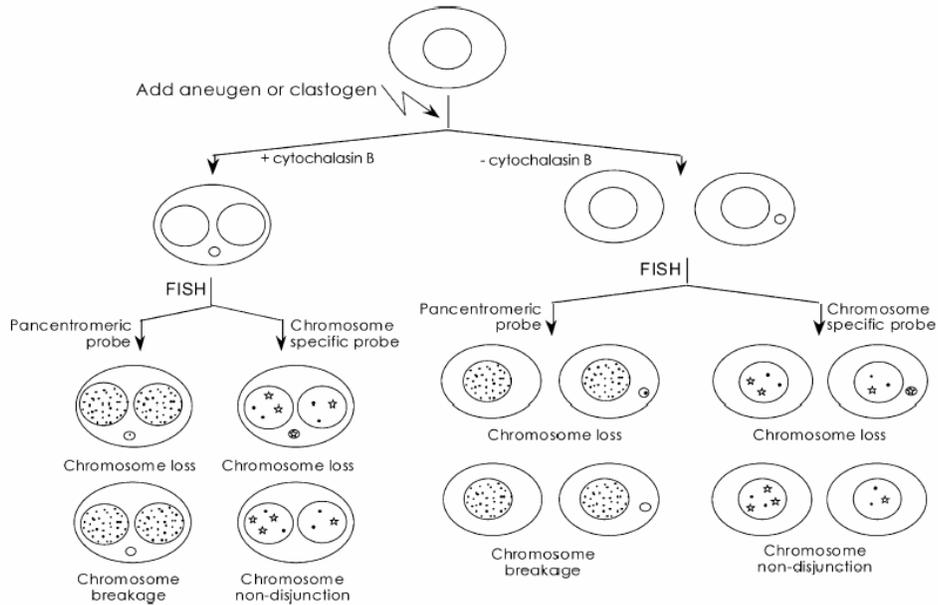


Fig. 4. Chromosome non-disjunction versus chromosome loss/breakage; (●): pancentromeric probe, detecting centromeres of all chromosomes, ★: chromosome specific pericentromeric probe for chromosome 1, (●) chromosome specific pericentromeric probe for chromosome 17 [29].

2.2. Comet assay

2.2.1. Internal standard

The internal standard for the Comet assay developed in the laboratory for Cell Genetics consists of untreated and ethylmethane sulphonate treated K562 cells. The internal standard was evaluated by (1) determining the different levels of variability that can influence the internal standard (different cultures, slides, donors, cell types, electrophoresis runs, scorers); (2) evaluating the variability of the researchers and electrophoresis runs in the quantification of the DNA damage of the internal standard implemented in 2 biomonitoring studies and an *in vivo* experimental study; (3) proposing an adequate mathematical system to integrate the internal standard in the data of test samples.

2.2.2. Biomonitoring: use of more specific reporter cells

Several protocols for using buccal mucosa cells were tested, but the cells always exerted a high percentage of DNA damage, probably due to the presence of DNases. In the future, when using these cells, a protocol developed in our laboratory for the Comet assay on mouse columnar epithelial colon cells will be used (which also showed the same problem using several other protocols).

2.2.3. Use of the Comet assay in the Global Repair Phenotype assay

For the study of the repair kinetics of control and exposed workers, 2.8ml of blood per donor (10 control individuals and 10 nuclear workers) was stored at 4°C in the dark until the following day. For each individual, 2 whole blood cultures of 35ml were prepared. The blood was placed in culture flasks at 37°C in Ham's F-10 medium

supplemented with 15% FCS. The lymphocytes were stimulated with 2% PHA. After 24 hours, cells were exposed to ^{60}Co γ -rays in the G1-phase of the cells cycle. The blood cultures were irradiated *in vitro* with 2 Gy at 0.1 Gy/min in a water bath at 0°C to prevent any repair. Irradiation was performed on whole blood to better mimic the *in vivo* situation. Dosimetry was performed at the position of the samples with a NE2571 cylindrical ionization chamber and NE2570 dosimeter (Nuclear Enterprises, Reading, UK), applying the IAEA 1987 Code of Practice. Aliquots (2x5ml) were removed from the cultures before and immediately after exposure and the Comet slides were stored in lysis solution at 4°C with internal standards prepared on the same day. The remaining blood cultures (25ml), having been kept on ice, were incubated at 37°C to allow acclimatization for 20 minutes. This time was established in preliminary experiments. Thereafter, repair assessments were performed after *in vitro* irradiation (0 minutes) at times 5, 10, 30, 60 and 120 minutes, aliquots of blood being centrifuged at 400 *g* for 10 minutes. Cells in 0.8% low melting point agarose was layered on top of an ordinary microscope slide that had been precoated with 1% normal melting point agarose. The slides prepared at different sampling times, all originating from the same individual, were kept overnight at 4°C in lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM Na₂-EDTA, pH10) supplemented just before use with 10% DMSO and 1% Triton X-100, with those obtained before and immediately after irradiation and internal standards prepared on the same day. They were processed together in the same electrophoresis session the following day.

One hundred cells per donor from 2 cultures were randomly captured on coded slides using a Leitz fluorescence microscope (x25 objective) coupled to a CCD camera and image analysis system (Komet 3.0, Kinetic Imaging, Liverpool, UK). The length of the comet tail in μm (=TL), the percentage of DNA in the tail (=TDNA) and the product of TL and TDA in μm (=tail moment or TM) were recorded. The initial DNA damage and residual DNA damage were calculated as follows (Leprat *et al.*, 1998):

Initial DNA damage = DNA damage immediately after *in vitro* irradiation - DNA damage in control cells before *in vitro* irradiation;

Per cent residual DNA damage at time *t* after irradiation (%RD) = $100 \times (\text{DNA damage at time } t \text{ after irradiation} - \text{DNA damage in control cells before irradiation}) \div (\text{DNA damage immediately after irradiation} - \text{DNA damage in control cells before irradiation})$. This residual damage was calculated for all 3 parameters (%RDTDNA, %RDTL, and %RDTM). This work was published in 2002 by Touil *et al.*

2.2.3. Influence of folic acid on DNA damage and repair

Lymphocytes isolated from venous blood were resuspended at 10^5 cells/ml and stimulated to divide in medium containing 2% phytohaemagglutinin. All cell cultures were allowed to grow in either normal RPMI medium containing 1 $\mu\text{g/ml}$ folic acid or in folic acid supplemented RPMI (4, 6, and 10 $\mu\text{g/ml}$). The effect of folic acid supplementation on styrene oxide induced DNA damage and repair was investigated in lymphocytes cultured up to 48 hours in the presence of different folic acid concentrations, using the Global Repair Phenotype assay. To induce DNA damage, human lymphocytes were washed once in PBS pH7.4 before exposure to 0.1mM styrene oxide for 1 hour. After one hour of styrene oxide treatment the cells were washed in PBS and either resuspended immediately in agarose for comet analysis or further incubated for 6 or 24 hours to allow repair.

3. RESULTS AND/OR RECOMMENDATIONS

3.1. Micronucleus test

3.1.1. Use of more specific reporter cells

We were able to obtain nasal cells in a non-invasive way and these cells could be cytospun unto microscopic slides. The staining of the slides however, is not optimal yet.

3.1.2. Micronucleus test for biomonitoring

Inclusion of measures of (1) micronuclei in mononucleated cells, (2) micronuclei in binucleated cells, (3) apoptotic cells, and (4) necrotic cells, in the CBMN assay, will lead to a more comprehensive assessment of DNA damage (Kirsch-Volders and Fenech, 2002).

3.1.3. Towards validation of the in vitro micronucleus test: Recommendations

The conclusions of the 1999 Washington work group, chaired and published by Micheline Kirsch-Volders, are given here:

Cells: the choice of cells is flexible, although this choice must be accounted for and one should take into account doubling time, spontaneous frequency of micronuclei and genetic background.

Slide preparation: the used fixation method must preserve the cytoplasm and its boundaries and must limit clotting. The use of fluorescent DNA specific dyes is advised for a better detection of smaller micronuclei.

Analysis: a micronucleus should have a diameter not exceeding one third of the diameter of the main nucleus, and it should not overlap with this main nucleus. In the cytokinesis-blocked method, the binucleated cells that are selected for analysis should contain 2 clearly separated main nuclei. Cells that undergo apoptosis should not be analyzed for the presence of micronuclei, since in this case they may have arisen through nuclear fragmentation caused by the apoptotic process.

Toxicity: cytotoxicity can be measured using different methods like cell growth, cell counts, nuclear division (i.e. proportion of binucleates), and division or proliferation indices. The majority of the work group advised that the highest concentration should induce at least 50% cytotoxicity.

Cytochalasin B: the use of cytochalasin B is an item of discussion. For human lymphocytes the use of cytochalasin B is advised (6 µg/ml for lymphocytes in whole blood cultures, and 3-6 µg/ml for lymphocytes in isolated lymphocyte cultures). For cell lines, the use of cytochalasin B is optional, since no definitive results on the pros and cons of using cytochalasin B were available at the time. Further studies were needed to clarify this issue.

Number of doses: at least 3 concentrations should be analyzed for the presence of micronuclei.

Treatment and harvest times: at the time insufficient data were available to define these times. Comparison to the principles of the *in vitro* metaphase test, a short treatment followed by repair (and thus absence of the compound) was agreed upon, as was a long treatment schedule (with or without repair period). The treatment should ideally cover several cell cycle stages.

The major recommendations of the 2002 Plymouth work group, chaired and published by Micheline Kirsch-Volders, concern :

Demonstration of cell proliferation: both cell lines and lymphocytes can be used, but demonstration of cell proliferation in both control and treated cells is compulsory for the acceptance of the test.

Assessment of toxicity and dose range finding : assessment of toxicity should be performed by determining cell proliferation, e.g. increased cell counts (CC) or population doubling (PD) without cytochalasin-B, or e.g. cytokinesis-block proliferation index with cytochalasin-B ; and by determining other markers for cytotoxicity (confluency, apoptosis, necrosis) which can provide valuable additional information.

Treatment schedules for cell lines and lymphocytes.

Choice of positive controls: without S9 both a clastogen (e.g. mitomycin C or bleomycin) and an aneugen (e.g. colchicin) should be included as positive controls and a clastogen such as cyclophosphamide when S9 is used.

Duplicate cultures and number of cells to be scored.

Repeat experiments: in lymphocytes, for each experiment blood from 2 different healthy young and non-smoking donors should be compared. In cell lines, the experiments needs only to be repeated if the first one is negative.

Statistics: statistical significance should not be the sole factor for determining positive results. Biological meaning should serve as a guideline. Examples of statistical analyses are given.

3.2. Comet assay

3.2.1. Internal standard

The mathematical model calibrating the test value with the negative control value (untreated K562) is considered to be the most useful, because the negative internal standard is more stable considering different electrophoresis runs and different scorers.

Percentage of DNA in the tail (TD) is the most suitable parameter for the analysis of DNA damage because of its lower inter-electrophoresis and inter-scorer variation as compared to tail length (TL), especially when the negative internal standard is used. This work was published in the course of this OSCT project (De Boeck *et al.*, 2000).

3.2.2. Use of more specific reporter cells

We were able to obtain nasal and buccal mucosa cells in a non-invasive way. Applying the Comet assay technique on these cells was very hard (the cells showed a high percentage of DNA damage, no matter which protocol for the technique was used).

3.2.3. Use of the Comet assay in the Global Repair Phenotype assay

For all individuals studied in the described biomonitoring study (Touil *et al.*, 2002) a statistically significant increase in the comet parameters (TL, TDNA and TM) in PHA-stimulated lymphocytes was detected immediately after *in vitro* exposure to 2 Gy γ -rays as compared with the DNA damage before irradiation. The initial damage for the three comet parameters was the same in both populations (control individuals and nuclear workers).

The most striking observation when using the Global Repair Phenotype assay was that compared with lymphocytes from controls, lymphocytes from exposed individuals showed a higher percentage of residual TL and TM after the 120 min repair time point, suggesting better repair in the controls. Despite these differences in repair between groups, there were no statistically significant differences between groups.

Before drawing any conclusions, it will be necessary to reanalyze DNA damage and repair phenotype in a larger group of nuclear workers taking into consideration smoking habit.

The Global Repair Phenotype assay can also be used at the individual level aiming at detecting individuals who have an elevated or diminished repair capacity in lymphocyte compared with an average level of repair. In addition, characterization of this capacity by defined parameters allows comparison between individuals. The data in our study suggest that people exposed to low chronic low level IR have more efficient repair than the controls.

3.2.3. Influence of folic acid on DNA damage and repair

There was no statistically significant influence of folic acid concentration on DNA damage as measured by the Comet parameters. There were equally no significant differences in comet parameters between repeated experiments (inter experimental variation) as well as between different donors (intra-individual variability). Important differences in repair were noted, with the culture medium with higher folic acid concentrations generally repairing better than in normal RMPI.

4. DISSEMINATION AND VALORISATION

4.1 Micronucleus test

4.1.1. Use of more specific reporter cells

No results to disseminate and valorize were available from this study.

4.1.2. Micronucleus test for biomonitoring

Four papers on the micronucleus test for biomonitoring were published in the framework of the Human MicroNucleus Project of which Micheline Kirsch-Volders is one of the 5 coordinators (Fenech *et al.*, 1999, Bonassi *et al.*, 2001, Fenech *et al.*, 2002, and Bonassi *et al.*, 2003).

4.1.3. Towards validation of the *in vitro* micronucleus test

While many publications applying the *in vitro* micronucleus test for genotoxicity studies and defining criteria for scoring (Fenech, 2000) came out, no international agreement was reached before on adequate protocols for performing the test in primary cells or cell lines. The discussions at the IWGT workshops on the *in vitro* micronucleus test held in Washington (1999) and Plymouth (2002) lead to discussions being taken to accurately perform a scientifically sound *in vitro* micronucleus test and these will support the redaction of a guideline by the OECD.

Two papers concerning the IWGT workshop recommendations on the *in vitro* micronucleus test appeared (Kirsch-Volders *et al.*, 2000 and Kirsch-Volders *et al.*, 2002).

4.2. Comet assay

4.2.1. Internal standard

The work on the internal standard was published in 2000 (De Boeck *et al.*, 2000) and has already been referred to in 1 publication in 2001 and 1 in 2002.

4.2.2. Use of more specific reporter cells

No results to disseminate and valorize were available from this study.

4.2.3. Use of the Comet assay in the Global Repair Phenotype assay

The work on the Global Repair Phenotype assay in a biomonitoring study concerning chronic low level exposure to ionizing radiation was published in 2002 (Touil *et al.*, 2002).

4.2.4. Influence of folic acid on DNA damage and repair

Results were obtained, but they are not sufficient yet for publication.

5. BALANCE AND FUTURE PERSPECTIVES

We are now very close to international implementation of the *in vitro* micronucleus test and the Comet assay, and we also have other techniques in hand so that in the near future we could investigate and possibly correlate repair phenotypes and genotypes to results obtained in the micronucleus test and the Comet assay in order to be able to make predictions about susceptibility. This work will be performed in the framework of one of our EU funded projects.

ACKNOWLEDGEMENTS

The work performed for this Belgian Science Policy project was performed in close collaboration with our EU funded projects (ENV4-CT97-0471 and QLK4-CT-2000-00058) and the HUMN project (QLRT-2000-00628).

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PART IV

THE MICRONUCLEUS ASSAY, VALIDATION, NORMALISATION AND QUALITY ASSURANCE

1. INTRODUCTION

The micronucleus assay is an assay for the evaluation of chromosomal damage in peripheral blood lymphocytes as a result of the exposure of an individual to mutagenic factors. It is as such a biomarker for a biological effect linked to cancer. The technique allows biomonitoring of large populations and is applied at large scale. In this way different populations with genetic syndromes and systemic diseases were already studied for the validation of the assay as indicator of health effects.

From point of view of governmental policy the availability of sensitive bioindicators of the exposure to cancerogenic and mutagenic factors is of great importance. Up to now the largest problem for the interpretation of the results of large scale studies with the micronucleus assay was the difference in the protocols used for the assay and the large intra- and interlaboratory differences. Up to now the lack of a standardised protocol for the execution of the test and the reporting of the results and the lack of the documents allowing a reliable quality assurance were a serious drawback for the legal value of the assay.

In the framework of the Programme for Scientific Support to Normalisation and Technical Regulations of the Belgian Science Policy, we have executed a scientific programme with the aim the normalisation and validation of the micronucleus assay and the elaboration of a reliable quality assurance system. A number of working groups on a world wide scale are also dealing with these matters : the international ISO working group ISO/TC 85/SC2 on "Standard criteria for service laboratories performing biological dosimetry by cytogenetics- (Quality assurance and control, evaluation of performance" (coordinator Ph. Voisin IPSN Fontenay aux Roses) and the HUMN (Human MicroNucleus project) working party on the normalisation of the micronucleus assay (coordinator M. Fenech CSIRO Adelaide Australia). The research units of the Universities of Gent and Brussels involved in present research project are participants in these working groups.

Normalisation of the test means in the first place the determination of a standard working procedure for the assay. Subsequently the test following this protocol has to be validated and finally procedures for quality assurance have to be worked out. In the execution of these tasks one has to take into account as much as possible the results of the international working groups.

The results of this work may lead to a validation file for the recognition of the micronucleus assay as method for the biomonitoring of populations exposed to mutagenic factors and for the accreditation of the laboratories performing the assay.

2. METHODOLOGY

In the execution of the different steps in the scientific work, the following order was kept on. In collaboration with the HUMN working party a detailed standard protocol was elaborated for the micronucleus assay as test for toxicity. Using this protocol the intrinsic variability of the assay as well as the intra-individual and inter-individual variability of the results of the test after an in-vitro irradiation with gamma rays as test

model were studied. Furthermore the distribution of the spontaneous micronucleus frequency in a non-exposed control population was determined, an important point with respect to the sensitivity of the assay. Subsequently a number of important elements in the validation file were worked out. More specifically, these elements concern: the confidentiality of personal information, the laboratory safety requirements, the calibration procedure for the cytogenetic evaluation, the scoring procedure, the criteria for converting a measured cytogenetic result in a dose estimate and the reporting of the results. Finally, attention was paid to the quality assurance and control of the micronucleus assay and results.

3. RESULTS

3.1. Elaboration of a detailed protocol for the micronucleus assay

The final standard protocol obtained after optimization is given below:

- Addition of 0.3 ml blood withdrawn in an heparinised tube to 4.5 ml culture medium consisting of RPMI 1640 medium (Gibco), Phytohaemagglutinin P (60 µg/ml, Difco) as mitogen and 10 % foetal calf serum (Gibco).
- Incubation in a CO₂ incubator at 37° C for 70 h in an atmosphere containing 5 % CO₂
- Addition of Cytochalasin B (Sigma) in a concentration of 6 µg/ml 24 h after the start of the incubation
- After an incubation period of 70 h cells are collected by centrifugation during 10 min at a speed of 1000 RPM and treated with a cold hypotonic solution of 0.075 M KCl .
- Subsequently cells are collected by centrifugation during 10 min at a speed of 1000 RPM and fixed a first time in a methanol-acetic acid -Ringer solution at a ratio of 10 over 1 over 11.
- The next day a second and a third fixation with a methanol-acetic acid solution 10 over 1 is performed.
- After centrifugation the supernatans is aspirated and the cell pellet is dissolved in 0.5 ml fixation fluid. After treatment with a vortex 60 µl of the cell suspension is dropped gently on a slide. After drying, cells are stained with a Romanowsky-Giemsa solution.
- Scoring of the number of micronuclei in the cytoplasm of the binucleated lymphocytes under the light microscope.

This protocol is the result of an investigation of different variables by the research group.

A first variable thoroughly investigated is the effect of culture time on the number of mononucleated, binucleated and polynucleated cells. The fractions mononucleated, binucleated and polynucleated cells on the slides were evaluated for culture times of 54 h, 64 h and 70 h. This study showed that 70 h is the optimal culture time and that shorter times result in a reduced yield of binucleates.

For mitogen stimulation of the lymphocytes purified phytohaemagglutinin of Difco (PHA P) gave better results than phytohaemagglutinin of Wellcome and this predominantly because of the reduced agglutination of lymphocytes when using purified phytoagglutinin.

A second variable, which was optimised was the ratio methanol-acetic acid for the fixation of the cells. Different proportions were tried out in the range 1 over 6 up to 1 over 30. A ratio of 1 over 10 gave the best result. Another important practical point for this fixation is the continuous use of the vortex during the fixation to avoid cell clustering.

A third part of the protocol, thoroughly investigated for optimization, is the nuclear staining method. Different possibilities were tried out. More general dyes as haematoxylin, thionin as well as more specific nuclear dyes as gallocyanin, Feulgen and CAS (Becton-Dickinson) gave a staining with a relatively low intensity. The best nuclear staining was obtained with the Romanowsky-Giemsa solution based from one side on the blue cathionic Azur B dye and from the other side on the red-orange anionic eosin Y dye. The DNA of the nucleus is stained metachromatic purple and the RNA-rich cytoplasm orthochromatic blue.

In the framework of the HUMN international working party the scoring criteria for the binucleated cells and the micronuclei were worked out in detail.

Scoring criteria for the binucleated cells

- Cells with two round or oval nuclei
- The two nuclei must be in the same condensation state
- The two nuclei must have similar size
- The two nuclei can touch each other or partially overlap
- The cytoplasmic boundary of the cell must be clear and intact
- The two nuclei may be attached by a fine nucleoplasmic bridge

Criteria for the identification and scoring of micronuclei in binucleated cells

- Morphologically identical but smaller than nuclei
- Round or oval in shape
- Diameter between 1/3 and 1/16 of the nucleus
- Nonrefractile
- May overlap or touch the main nucleus
- Same color as nucleus
- Similar staining intensity as nucleus

A very important point of discussion in the scoring criteria at an international level was the acceptance of micronuclei when they overlap the main nucleus. A large comparative study between micronucleus frequencies scored with the criterion that the micronuclei may not touch the main nucleus and with the criterion that overlapping and touching micronuclei are also scored showed no significant differences in variability indicating that both criteria are equivalent. With the criterion that also overlapping and touching micronuclei are scored a higher statistical accuracy is obtained. Therefore this criterion was adopted in this project.

3.2. Intrinsic validation of the micronucleus assay

To investigate the reproducibility of the technique starting from the same material a blood sample of a donor was irradiated in vitro with 3.5 Gy ⁶⁰Co gamma radiation. Starting from this blood sample six cultures were started. Application of the elaborated culture- and scoring protocol resulted in micronucleus frequencies ranging

between 747 and 831 micronuclei per 1000 binucleated cells with a mean value of 791 and a standard deviation of 39 micronuclei per 1000 binucleated cells or a variability of 5 %. This proves the reproducibility of the assay.

As a next step the intra-individual variation in the micronucleus frequency after in vitro exposure to a mutagen was studied. As model again an in vitro irradiation of a blood sample with 3.5 Gy ^{60}Co gamma radiation was used. Seven blood samples were taken from two donors over a period of nine months and the micronucleus frequency was determined after in vitro irradiation of the samples. The results are summarised in the Table below.

Table: Overview of the number of micronuclei per 1000 binucleated cells induced by irradiation of blood samples of two donors withdrawn over a period of nine months

Date bloodsampling	Donor 1	Donor 2
1/27/2000	826	769
1/31/2000	907	815
2/8/2000	922	845
2/17/2002	849	764
3/20/2002	980	724
9/10/2002	786	647
10/10/2002	706	700
Mean value	854	752
Standard deviation	92	68

These results show a larger variation for the two donors, 11% and 9 % respectively, compared to the intrinsic variability of the test: 5 %. The larger variation on the results obtained for the blood samples taken over a period of nine months can be explained by the influence of physiological factors in a blood sample which may vary in an individual. As the sample material consists of peripheral blood lymphocytes the immunological status of the donor may play a role. Also hormone levels and diet can influence the result of a cytogenetic test as the micronucleus assay.

Finally, we investigated the value of the micronucleus assay as exposure biomonitor after exposure of an individual of a population to a mutagen. The value and the use of a cytogenetic test as exposure biomonitor depends to a large extent on the inter-individual variation. The smaller this variation after a single well defined exposure the higher the value of the test as exposure biomonitor. For this study we used again an in vitro irradiation of blood samples with 3.5 Gy ^{60}Co gamma rays as model. A blood sample was taken from fifteen donors of all ages and both sexes. Application of the micronucleus assay resulted in values for the micronucleus frequency ranging from 652 up to 935 micronuclei per 1000 binucleated cells with a mean value of 806 and a standard deviation of 73 micronuclei per 1000 binucleated cells. The inter-individual variation at the considered exposure amounts to 9 %, almost the same value as the intra-individual variation.

These studies led to the conclusion that the inter-individual differences in dose-response are of minor importance in the application of the micronucleus assay as exposure biomonitor. The physiological factors in a blood sample result also in a variation of about 10 % in the micronucleus frequency for blood samples withdrawn

at different times for the same donor. This is about the same value as observed for the variation on the micronucleus frequency between different donors after the same exposure.

The results of this study of the reproducibility of the micronucleus assay and the intra-individual and inter-individual variation were published and are more described in detail in the international journal Radiation Research (vol. 157 pp.472-477, 2002).

3.3. Study of the spontaneous micronucleus frequency in a non-exposed control population

For the application of the micronucleus assay as exposure monitor the distribution of the spontaneous micronucleus frequency in a non-exposed control population is an important point with respect to the sensitivity of the test. Apart from the determination of the parameters of the distribution as mean value and standard deviation, also the factors affecting the spontaneous micronucleus frequency have to be identified.

For this purpose we performed a study of the micronucleus frequency in a control group of 23 men and 37 women, occupationally not exposed to mutagenic factors. The participants of the study signed a written informed consent and filled in a questionnaire. Gender, date of birth, smoking habits, work-related exposure to hazardous non-mutagenic agents, previous exposures to diagnostic X-rays as a patient, nuclear medical examinations and the use of therapeutic drugs were registered. For these individuals the standard protocol of the micronucleus assay was applied. This study was performed in a strong collaboration with the occupational medical service of the University Hospital Gent.

The mean micronucleus frequency for the studied population with mean age of 41.7 years (27-61 years) amounted to 18.63 with a standard deviation of 7.53 per 1000 binucleated cells. Within the population a strong age dependence of the spontaneous micronucleus frequency was observed. A linear regression analysis showed an increase of 0.52 micronuclei per year ($r = 0.42$). Another important factor for the spontaneous micronucleus incidence is the donor gender. The number of spontaneous micronuclei in the female population is significantly higher than in the male population: 23.54 versus 16.43 micronuclei per 1000 binucleated cells. The increase with age is also larger in the female population than in the male population: 0.58 versus 0.44 micronuclei per year. In general, the spontaneous micronucleus frequency is 1.4 times higher in women compared to men. This gender effect on the micronucleus frequency seems to be due to the higher sensitivity of the inactive x-chromosome for malsegregation in mitosis compared to the autosomes.

In the study we investigated also if the smoking habit of the individual has an effect on the micronucleus frequency. A comparison between smokers and non-smokers in the group shows a small enhancement of the number of micronuclei in the group of smokers : 21.13 versus 19.87 micronuclei per 1000 binucleated cells. However, application of the Wilcoxon test to the data shows no statistically significant difference. An analysis of the data according to the number of cigarette-years and the number of cigarettes per day shows also no significant effect on the number of micronuclei.

The data of this study represent a data bank of a control population, which can be used for the statistical analysis of the results of the micronucleus assay applied to populations exposed to mutagenic factors. The results summarized in this section are

more described in detail in a paper in the international journal Mutagenesis (vol. 15 pp.245-249, 2000).

3.4. Elements of the validation file of the micronucleus assay as biomonitor

In the period 2000-2002 a working group of the International Standardisation Organisation (ISO) worked out a draft for an ISO-standard for laboratories performing biological dosimetry with cytogenetic techniques. As representative of Belgium we participated to this working group and were heavily involved in the scientific work leading to the draft. The final result of the working party is a document entitled "Standard criteria for service laboratories performing biological dosimetry by cytogenetics- (Quality assurance and control, evaluation of performance)" with nr. ISO/TC 85/SC 2 551. At the moment this document is distributed for voting to the ISO representatives of the different nations involved in the organisation.

In accordance with this proposal of ISO standard a number of elements in the validation file of the micronucleus assay as biomonitor for mutagenic compounds and physical factors were worked out.

Confidentiality of personal information

The confidentiality of the personal information of the individual under study has to be maintained. This includes the identity, medical data and social status. The laboratory needs to have established protocols for maintaining the anonymity of the blood samples. To avoid the identification of the individual while guaranteeing the traceability of the analysis, the blood samples should be coded upon arrival at the laboratory by an authorized person. Persons with this authority shall have signed a commitment to confidentiality regarding their duties within the laboratory. Apart from the coding also the decoding, the interpretation of the results of the micronucleus assay and the report are also to be performed by an authorized person. During the execution of the assay the anonymity of the samples has to be maintained.

The request for an analysis should be made by an authorized person. In the case of exposures to mutagenic factors at the working place this person is a doctor of the occupational medical service of the individual involved. He/she receives also the report with the results of the analysis. In any case the blood sampling for the micronucleus assay has to be preceded by the individual's signed informed consent.

The reporting of the results and the documents with respect to the personal data of the individual must be stored in the laboratory in a place only accessible to the authorized persons for at least 30 years for possible medico-legal re-evaluation of the case.

Laboratory safety requirements for the execution of the micronucleus assay.

The safety requirements include microbiological, chemical and optical considerations.

Handling human blood poses risks of infection of the technical staff. All blood samples should be regarded as being potentially infectious even if they are known to be derived from apparently healthy persons. Specimens must be unpacked and manipulated in a class 2 microbiological safety cabinet. Vaccination of the staff against hepatitis B is necessary. The legal and ethical aspects of HIV testing of blood

samples upon receipt have to be considered for prevention of the infection of the laboratory staff.

Regular control of cultures and equipment for microbiological infections has to be performed. All biological waste must be collected separately as risk-bearing medical waste.

Execution of the micronucleus test presents no serious exposure of staff to chemical hazards as they are mostly used in small volumes and low concentration dilutions. The main reagents of concern and their internationally agreed risk phrases (R numbers) are listed below:

- Benzylpenicillin (R42,43)
- Cytochalasin (R26,27,28,63)
- Giemsa stain (R20,21,22,40,41)
- Heparin (R36,37,38)
- Phytohaemagglutinin (R20, 21, 22, 43)

with R20 harmful by inhalation, R21 harmful in contact with skin, R22 harmful if swallowed, R26 very toxic by inhalation, R27 very toxic in contact with skin, R28 very toxic if swallowed, R40 possible risks of irreversible effects, R41 risk of serious damage to eyes, R42 may cause sensitisation by inhalation, R43 may cause sensitisation by skin contact, R63 possible harm to the unborn child.

The necessary measures have to be taken to avoid the exposure of staff to the ultraviolet light used in sterilising the interior of microbiological safety cabinets and for exposure of slides in staining procedures.

A safety plan has to be available and written safety procedures for protection against microbiological , chemical, and optical hazards have to be present.

Calibration curve

For the interpretation of the results the laboratory has to define in vitro dose-response curves with respect to the micronucleus frequency for the mutagens under study. The dose-range in these in vitro experiments has to cover the doses to which the individuals or the population has been exposed. In the case of exposure to ionising radiation the calibration curve has to cover the dose-range 0-4 Gy. Special attention as to be paid to the low dose region. For each mutagen the minimum detectable level has to be determined. In the case of exposure to ionising radiation this level is as low as 100 mGy.

The inter-individual variability in the dose-response has to be determined . Reverse, from the measured micronucleus frequency these data allow the determination of the uncertainty on the dose estimate to which the individual has been exposed in vivo. In general this uncertainty is expressed as 95% confidence limits. The experimental data used in the calibration of the micronucleus test as toxicity assay have to be specified in the quality handbook.

Scoring procedure

The scoring has to be performed by laboratory staff with experience and training in micronucleus scoring. Intercomparisons between scorers in the laboratory have to be

organised on a regulatory base. It is recommended that the scoring is performed by two scorers, scoring an equal amount of binucleated cells. At the same time it is recommended that different slides are taken into account. In case a computerised image analysis system is used in the selection of the binucleated cells and the scoring of the micronuclei, the system has to be validated by a comparative study with manual microscopic scoring.

Reporting of results

The report of the analysis has to include the following elements:

- The identification of the exposed subject : name or code of the subject, date of birth
- Description of the case: all information provided by the customer that is relevant to the interpretation of the result shall be stated.
- Data regarding the request: name and address of the inquirer, date of order, the reason for the order.
- Description of the results of the micronucleus assay: date of blood sampling, date of its arrival in the service laboratory, micronucleus frequency and number of binucleated cells scored.
- Interpretation of the results depending on the circumstances: a dose estimate for the considered mutagen for the exposed individual including a quantification of the uncertainties. The level of the spontaneous micronucleus frequency used for the interpretation and the used in vitro dose-response curve for the conversion of the micronucleus frequency to the dose have to be included. A statement is necessary on whether the dose estimate was made assuming acute or chronic exposure and how protraction had been accounted for.

3.5. Quality assurance and quality control

Quality assurance

The fundamental requirements for a full quality assurance program include:

- Compliance with general operational requirements stated in approved written procedures
- A well documented in-house quality assurance programme
- Periodic performance evaluations by internal and external intercomparisons and on-site expert assessments
- Completely documented procedures for services provided to customers

The quality assurance programme must assure the quality of the laboratory's output over extended periods of time. The four requirements as described above provide the strategy for safeguarding the quality of the result of the micronucleus assay and the interpretation. The laboratory has to provide a quality handbook with information on the structure of the organisation, the qualification of personnel, the description of the used consumables and equipment, a detailed description of the used protocol and the procedures for quality assurance.

Periodic on-site expert evaluations are necessary to assure that the laboratory is capable of yielding services that meet the technical quality specifications i.e. a

reliable determination of the micronucleus frequency with a sound interpretation. Proficiency tests periodically evaluate measurement consistency with certified or suitably qualified cytogenetic laboratories. Analyses are carried out by the reference and the testing laboratory and comparisons are made between the values obtained by both laboratories.

A quality assurance plan includes the following: organisation structure, qualification and training of personnel, procurement of materials, chain of custody for control of materials and samples, inspection and testing of material and equipment, control and maintenance of standards, quality assurance records.

Quality control

Performance checks shall be conducted to ensure that the analysis procedures and equipment as well as the results and interpretation are conform with predetermined operational requirements. For the micronucleus assay a number of specific performance checks has to be performed.

A performance check of sample transport integrity is necessary. In many cases blood collection occurs at sites distant from the processing laboratory and damage to the sample can occur during transportation. A minimum-maximum thermometer in the shipping container will provide information on the temperature range during transport. Special care has to be taken that the temperature is not exceeding 37 °C. If air transportation is used, one has to avoid the x-irradiation at the security checkpoints. A sheet of x-ray film should be included in the shipping package to verify this.

A system for recording the collection, transport and storage of the blood samples has to be worked out in order to guaranty sample integrity. As internal quality assurance, negative controls from unexposed individuals and internal positive controls have to be included in the study to prove the reliability of the whole procedure. Blood from the exposed and the control individuals has to be handled in the same way. Samples of both groups have to be processed concurrently and not successively. The use of coded samples is critical to avoid bias in the scoring.

Performance checks of equipment has to be performed at a regular base. Especially the stability of the temperature control of the incubators has to be controlled. Replicate in vitro measurements should be made periodically.

A number of performance checks of the micronucleus assay protocol have to be performed as quality control procedures. It can be useful to prepare a slide for differential count from each blood sample before starting the cultures. It is recommended that the same lot of media and reagents be used throughout a study. Culture, fixation and staining procedures as well as the composition of all reagents must be described in detail in the quality handbook. Replicate samples should be processed periodically. The number of quality control samples shall be at least 5 % of the total samples analysed.

Concerning quality control of scoring, a number of measures have to be taken. Within the set of slides a positive quality assurance standard should be included. Internal quality assurance involves a periodical comparison of the scoring results of replicate samples between scorers. External quality assurance involves the sharing of replicate samples with other laboratories and the organisation of multicenter intercomparisons at regular time intervals.

For the statistical analysis of the data non-parametric tests are indicated. The results of the negative and positive internal quality assurance controls are used to demonstrate the reliability of the methodology and scoring.

4. DISSEMINATION AND VALORISATION

The project allowed us as representative of Belgium in the International Standardisation Organisation (ISO) to contribute substantially to the elaboration of the ISO/TC 85/SC 2 n 551 document on "Standard criteria for service laboratories performing biological dosimetry by cytogenetics- (Quality assurance and control, evaluation of performance". A lot of the work done on the validation and quality assurance of the micronucleus assay in the framework of present project is included in the standard after discussion with the ISO working group. The draft of the ISO standard is now open for voting by the members of the different nations involved in the ISO.

The work done on the standardisation of the protocol including the scoring allowed us in the same way to participate in the Human MicroNucleus (HUMN) international working group. This collaboration made it possible to give the results of present project an international dimension. Scientific papers with a summary of the results of the HUMN project appeared in 2001 and 2002. These papers deal with the effect of laboratory protocol, scoring criteria and host factors on the micronucleus frequency and the intra- and inter-laboratory variation in the scoring of micronuclei.

For dissemination of the results of present project in the scientific community also other papers apart from the paper on the HUMN project were published in international journals with PEER review and citation index. A list of the papers is given below.

The micronucleus assay is frequently used as exposure biomonitor in the case of exposures of individuals and populations to mutagenic compounds and ionising radiation. Especially in the case of putative overexposures there is a need for official recognition of the assay from legal point of view. The normalisation, solid validation and quality assurance worked out in present project are essential elements in the recognition file of the test as exposure monitor. To elucidate the micronucleus assay as normalised and validated instrument at the workplace, the applications of the test are discussed now in the educational programmes of environmental and occupational medicine. It is especially in this field that a reliable biomonitor for exposures is needed.

List of papers

S. Bonassi, M. Fenech, C. Lando, Y. Lin, M. Ceppi, WP. Chang, N. Holland, M. Kirsch-Volders, E. Zeiger, S. Ban, R. Barale, MP. Bigatti, C. Bolognesi, C. Jia, M. Di Giorgio, L.R. Ferguson, A. Fucic, O.G. Lima, P. Hrelia, A.P. Krishnaja, T-K Lee, L. Migliore, L. Mikhalevich, E. Mirkova, P. Mosesso, W-U. Muller, Y. Odagiri, M.R. Scarfi, E. Szabova, I. Vorobtsova, A. Vral, A. Zijno.

Human MicroNucleus Project: International Database Comparison for Results With the Cytokinesis-Block Micronucleus Assay in Human Lymphocytes: Effect of Laboratory Protocol, Scoring Criteria, and Host Factors on the Frequency of Micronuclei

Environmental and Molecular Mutagenesis 37: 31-45 (2001)

M. Fenech, S. Bonassi, J. Turner, C. Lando, M. Ceppi, WP. Chang, N. Holland, M. Kirsch-Volders, E. Zeiger, MP. Bigatti, C. Bolognesi, C. Jia, G. De Luca, M. Di Giorgio, LR. Ferguson, A. Fucic, O. Garcia Lima, P. Hadjidekova, P. Hrelia, A. Jaworska, G. Joksic, AP. Krishnaja, T-K Lee, A. Martelli, M.J. McKay, L. Migliore, E. Mirkova, W-U Muller, Y. Odagiri, TT Orsiere, MR Scarfi, M.J. Silva, T. Sofuni, J. Suralles, G. Trenta, I. Vorobtsova, A. Vral, A. Zijno.

Intra- and inter-laboratory variation in the scoring of micronuclei and nucleoplasmic bridges in binucleated human lymphocytes. Results of an international slide-scoring exercise by the HUMN project.

Mutation Research. Genetic Toxicology and Environmental Mutagenesis 534: 45-64 (2002).

H. Thierens, A. Vral, R. Morthier, B. Aousalah and L. De Ridder

Cytogenetic monitoring of hospital workers occupationally exposed to ionising radiation using the micronucleus centromere assay.

Mutagenesis 15: 245-249 (2000)

P. Voisin, F. Barquinero, B. Blakely, C. Lindholm, D. Lloyd, C. Luccioni, S. Miller, F. Palitti, P. Prasanna, G. Stephan, H. Thierens, I. Turai, D. Wilkinson and A. Wojcik.

Towards a normalization of biological dosimetry by cytogenetics.

Cellular and Molecular Biology 48: 501-504 (2002)

A. Vral, H. Thierens, A. Baeyens and L. De Ridder.

The micronucleus and G2-phase assays for human blood lymphocytes as biomarkers of individual sensitivity to ionising radiation: limitations imposed by intraindividual variability.

Radiation Research 157: 472-477 (2002)

5. PERSPECTIVES

The research performed in the framework of present project represents an important step towards the normalisation and validation the micronucleus assay . Further elaboration of the validation towards specific applications in environmental or occupational medicine will allow the recognition of the assay as biomonitor for individuals and populations. Laboratories executing the assay as described and presenting a validation file can then be accredited for the specific applications of the assay described in the accreditation scope. In view of the work performed by the ISO working group and the well-documented data on the micronucleus frequencies after exposure to X-rays and other ionising radiations official recognition of the micronucleus assay as biodosimeter in case of putative overexposures is a direct application of present project. The work performed in the framework of present project serves as a scientific base to attribute to the micronucleus assay a legal value as biomonitor.

Apart from the micronucleus assay, other cytogenetic techniques as the scoring of unstable and stable aberrations in metaphases of lymphocytes are also candidates for which normalisation, validation and quality assurance are necessary. These techniques have their specific applications in occupational and environmental mutagenesis and also here, there is an urgent need to work out a normalisation and validation research programme as has been done for the micronucleus assay in present project. Taken into account the analogy the obtained results can serve as an example for the other cytogenetic techniques.

Uitgegeven in 2005 door het Federaal Wetenschapsbeleid

De wetenschappelijke verantwoordelijkheid over de inhoud van dit eindverslag berust volledig bij de auteurs.

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