Biomarkers, January–February 2005; 10(1): 80–94

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MEETING REPORT

New and improved biomarkers ready to be used in health-risk oriented exposure and susceptibility assessments: report of the 6th International Symposium on Biological Monitoring in Occupational and Environmental Health[1]

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ISBM beer was brewed in Heidelberg, Germany, this year

Previous meetings were in Kyoto, Japan (1992), Parma, Italy (1994), Helsinki, Finland (1996), Seoul, South Korea (1998) and Banff, Canada (2001). Of the Banff meeting, a report has appeared (Scheepers and Heussen 2002). This year the meeting was hosted by the University of Heidelberg, Germany. It was prepared by the Scientific Committee on Occupational Toxicology (SCOT) and the Scientific Committee on Toxicology of Metals (SCTM). On the occasion of the meeting, the local brewery produced a batch of 'ISBM' beer; a limited edition probably consumed mostly during the conference dinner by the participants. The meeting attracted an interdisciplinary audience and showed a balance of exploratory research and applications in the field of public health.

Highlights were the presentations of new methods for the determination of secondary metabolites of di(2-ethylhexyl)phthalate (DEHP) and for haemoglobin adducts and mercapturic acid urinary metabolites of acrylamide. Both methods are of interest to evaluate exposures in the general population. Adducts of some industrial chemicals such as N,N-dimethylformamide, 2,4-toluene diisocyanate and 1,2-epoxy-3,4-butanediol to globin or albumin were characterized by mass spectrometry (MS). This technique has become an indispensable technique to support multi-element trace determinations of metals (induced coupled plasma MS) and complex organic products such as adducts to proteins (mostly by MS/MS). High-throughput nano liquid chromatography (nLC) systems hyphenated with various sophisticated

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ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500500050085

MS-detectors are the future perspective to speed up the detection and characterization of binding products to DNA and proteins. Apparently, to date not many research groups engaged in biological monitoring research have access to these instruments since only one paper was presented during the meeting. The climate for the application of existing and new methods biological monitoring in Europe is gradually improving. A definition of 'biological limit value' (BLV) has been adopted by a committee of experts assigned by the European Commission (Scientific Committee on Occupational Exposure Limits, SCOEL). This European Union committee has the possibility to propose new BLVs, e.g. for toxic substances absorbed by the skin. Results from ongoing laboratory inter-comparison schemes provide confidence in the analytical performance of research laboratories and an increasing number of routine laboratories.

Most new applications are powered by mass spectrometry

During the meeting, new or improved methods for biological monitoring were presented for more than 20 chemical substances (Table I). Most methods were validated in humans, either volunteers exposed in a controlled environment or workers and/or subjects in the general environment (experimental animals are not much used). Mass spectrometry (specifically MS/MS for organics and ICPMS for metals) appears to be the most used method of detection for the determination of biomarkers to date. There were some quite interesting contributions describing the use of biological monitoring to study phenomena such as polymorfisms in biotransformation enzymes but also to find answers to questions related to exposure and health risk assessment.

Creatinine causes headache

There is an on-going debate about the use of creatinine (a metabolite of creatine from muscle metabolism) to normalize/standardize for diuresis in urine samples (urinary metabolite concentrations are expressed as μmol metabolite per mole creatinine). If creatinine was excreted at a constant rate (as often assumed), it would be a suitable parameter to normalize metabolite concentrations for variations in urine density. It is obvious that within one subject an increase of muscle metabolism such as in physical exercise or consumption of boiled meat leads to higher creatinine values and thus to lower standardized urinary metabolite values. In sports, creatine monohydrate may be used as a supplement to increase performance, but it also results in an enhanced excretion of urinary creatinine. On a longer time scale, a decrease of the urinary creatinine level with age was observed. Overestimation of environmental exposures such as cadmium was demonstrated in a cohort of 11 000 never-smoking Japanese women, aged 20–85 years (M. Ikeda, Kyoto). A decreasing muscle volume and possibly lower intake of meat by elderly people may explain this trend. In daily practice, standardization is important in the case of a large difference in density due to high physical activity in sports or heavy work loads. This is also the case when the first urine after awakening is used as a pre-shift spot sample and an often more diluted post-shift urine sample. This difference is often used to estimate the contribution from uptake during the day for toxic substances with intermediate elimination half-lives (2–20 h). Using the calculated difference of post- and pre-shift urine without volume standardization could then lead to an underestimation of the uptake. In

Table I. New and improved biomarkers introduced at the 6th ISBM in Heidelberg.

Chemical substance	Toxicity	Biomarker	Method	Validation	Presented by
1-Alkoxy-2-propanols	irritant	1-alkoxy-2-propanols in urine	GC-FID	workers	J. Laitinen et al. (Kupio)
1,3-Butadiene	human carcinogen	1,2-epoxy-3,4-butanediol albumin adducts in serum	LC-MS/MS	*in vitro* studies	C. Lindh (Lund)
2,4- and 2,6-Toluene diisocyanate (TDI)	sensitizer	toluenediamine-valine-hydantoins in whole blood	GC-NCI-MS	workers	J. Mráz et al. (Prague)
1-Bromopropane	human neurotoxic, human reprotoxic	globin *S*-propylcysteine (haemoglobin adduct) in blood N-acetyl-*S*-propylcysteine (mercapturic acid) in urine	LC-MS/MS	rats	G. Ichihara et al. (Aichi)
3-Nitrotyrosine	marker of inflammation	3-nitrotyrosine in exhaled breath condensate	LC-MS/MS	volunteers	Th. Göen et al. (Erlangen)
2-Butoxyethanol (BE)	irritant, hepatotoxic	2-butoxyacetic acid in urine	not specified	volunteer	S. Kezic et al. (Amsterdam)
4,4′-Methylenediphenyl diisocyanate (MDI)	sensitizer	4,4′-methylenedianiline in serum	GC-NCI-MS	workers	Y. Morita (Tokyo)
Acrylamide (AA)	animal carcinogen, neurotoxic	N-2-carbamoylethylvaline and N-(*R*,*S*)-2-hydroxy-2-carbamoyl-ethylvaline and (haemoglobin adducts) in blood	GC-NCI-MS	workers	T. Schettgen et al. (Erlangen)
		N-acetyl-*S*-(2-carbamoylethyl)-L-cysteine and N-(R/S)-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (mercapturic acids) in urine	GC-MS	general population	M. Boettcher (Erlangen)
Acrylonitril	human carcinogen	N-2-cyanoethylvaline (haemoglobin adducts) in blood	GC-MS/MS	workers	M. Bader (Hannover)
Anthracyclines	multi-resistance	anthracyclines in urine	HPLC	not specified	C. Minoia (Pavia)
Benzene	human carcinogen	*S*-phenyl cystein in blood	ELISA	workers	D. Fox (Chapel Hill)
Benzo[a]pyrene	human carcinogen	3-hydroxybenzo[a]pyrene (3-HBP)	HPLC-FD	general population	M. Lafontaine et al. (Vandoeuvre)
Ethylene oxide (EO)	human carcinogen	N-(2-hydroxethyl)valine in whole blood	immunoassay against GC/MS		L. Ball et al. (Cardiff)

Table I (*Continued*)

Chemical substance	Toxicity	Biomarker	Method	Validation	Presented by
Di(2-ethylhyexyl)-phthalate (DEHP)	animal carcinogen, animal reprotoxic	mono(2-ethyl-5-carboxypentyl)phthalate (5-carbo-MEHP), mono(2-ethyl-5-hydroxyhexyl)phthalate (5-hydroxy-MEHP), mono(2-ethyl-6-hydroxyhexyl)phthalate (6-hydroxy-MEHP), mono(2-ethyl-5-oxoheyxyl)-phtathalate (5-oxo-MEHP) and mono(2-ethylhexyl)phthalate (MEHP) in urine	LC-LC-MS/MS	volunteer	H. Koch et al. (Erlangen)
			GC-MS/MS	general population	H.-W. Hoppe (Bremen)
N-methyl-2-pyrrolidone (NMP)	irritant	5-hydroxy-N-methyl-2-pyrrolidone (5-HNMP), N-methyl-succinimide (MSI) and 2-hydroxy-2N-methylsuccinimide (2-HSMI) urinary metabolites	LC-MS/MS	volunteers	Carnerup et al. (Lund)
N,N-dimethyl formamide (DMF)	human carcinogen	N-methylcarbamoylvaline globin adduct in whole blood	GC-MS	workers	J. Mráz et al. (Prague)
N,N-dimethylacetamide	hepatotoxic	*S*-acetamido-methyl mercapturic acid in urine	LC-MS	workers	L. Perbellini et al. (Verona)
		N-methylacetamide in urine	GC-MS		
Naphthalene	animal carcinogen	1- and 2-naphthol in urine	online columns switching HPLC	workers and general population	R. Preuss et al. (Erlangen)
		S-(1-naphtyl)- and *S*-(2-naphthyl)cysteins from blood	ELISA	workers	D. Fox (Chapel Hill)
Phenanthrene	photosensitizer	1-, 2-, 3-, 4- and 9-phenanthrol in urine	HPLC-FD	workers	E. Elovaara (Helsinki)
(Synthetic) pyrethroids	neurotoxic	*cis*-DCCA, *trans*-DCCA, *cis*-DBCA, 3-BPA and FPBA (pyrethrum esters in urine)	HRGC-MS	volunteers	G. Leng (Leverkusen)
Pyrethrin	suspected endocrine disruptor	chrysanthemumdicarboxylic acid (*trans*-CDCA) in urine			
Toluene	neurotoxin	toluene in urine	GC-MS	general population and workers	S. Fustinoni et al. (Milan)
Xylylene diamine	sensitizer	xylylene diamine and hydrolysable metabolites in urine	GC-MS	workers	J. Cocker et al. (Buxton)

biostatistical analysis of biomonitoring data, it is recommended to analyse the data with and without a creatinine normalization of the urine levels. Adjustments for age, strenuous physical activity and meat intake is possible in the phase of statistical analysis if sufficient numbers of observation are available. For those involved in the setting of biological standards, creatinine complicates interpretation because most investigators used to report urinary excretion of metabolites using a normalization creatinine. Two recent papers review the use of creatinine in biological monitoring (Garde et al. 2004, Viau et al. 2004).

Which is the better biomarker for toluene exposure?

The unmetabolized toxic substance or its primary metabolite is not always the most suitable or practical biomarker for exposure. Several contributions dealt with the choice of the most suitable urinary metabolite to evaluate exposure to toluene. Toluene is extensively metabolized mostly to benzoic acid, followed by conjugation with glycine to hippuric acid (HA). For this metabolite, a high background is observed, perhaps due to its non-specificity for toluene. For example, benzoic acid, in Europe known as E210, E212 or E213, is a food preservative used in salads and meat products causing enhancement of the background values. There are also foods with a natural high content of benzoic acid such as wild berries (e.g. cranberries). The parent compound can be measured as well. A solid-phase micro-extraction (SPME)-based approach for the analysis of toluene from urine was presented by Dr S. Fustinoni (Milan, silvia.fustinoni@unimi.it). This biomarker discriminated well between rotogravure printing workers and reference subjects, based on the collection of spot urine samples at the end of the day (excretion half-life of about 2 h). A concern may be that it is important to prevent losses of the volatile toluene from the urine container. Apart from possible losses, there is also a risk of a contamination of the sample from the environment. The third alternative *o*-cresol (a minor metabolite with an excretion half-life of 3–5 h) produced similar results as toluene but showed an increase related to smoking. There is some discussion if this would significantly interfere in studies of workers' exposure. A fourth possibility is determination of *s*-benzylmercapturic acid (S-BMA) that was detected as a minor metabolite in volunteers. There was no apparent pattern of absorption or elimination corresponding to expected toxicokinetics. Perhaps this metabolic pathway becomes more relevant when other enzyme systems are saturated such as previously observed in toluene sniffers. Overall, *o*-cresol appears to be the most robust biomarker for exposure assessment, with toluene in end-exhaled air for semi-quantitative exposure assessments as an alternative (Dr K. Jones, Buxton, katherine.jones@hsl.gov.uk).

How good (or bad) is your breath?

Collection of exhaled air, which is also non-invasive, appears less popular probably because knowledge about the properties of toxic substances (kinetics of excretion) and their metabolites is needed for reliable sample collection and interpretation. For the laboratory technician who knows how to operate a thermal desorption unit mounted on a GC, breath provides a very clean matrix and an enormous potential of simultaneous determinations of volatile organic compounds (VOC).

VOC can be determined in the gas phase of exhaled air. Three different breath sample types were compared in a volunteer study by Dr Morgan (Seattle, mmorgan@u.washington.edu). Concentrations in alveolar air can be determined in so-called end-exhaled air (the last about 100 ml air expired after a normal inhalation) and by rebreathing (relaxed breathing into a flexible bag for 15 min). Both methods gave good estimates for venous blood levels. Collection of so-called mixed alveolar air gave lower values because a fraction (dead space volume), which does not equilibrate with blood in the alveoli, dilutes the sample resulting in values that underestimate venous blood levels. Corrections for this dilution are difficult to make. Collection of breath by rebreathing appears a reliable method for use in the laboratory, e.g. in human volunteer studies. Good agreement between the rebreathing and end-exhaled air have been obtained for halothane, 2-butanone, acetone, toluene and ethanol in volunteer studies, showing that both methods are reliable. In the field the use of rebreathing may be unpractical since the collection bag should be kept at a constant temperature (40°C, so-called isothermal rebreating) to prevent condensation of water and also to calculate venous blood concentrations from the venous blood — air partition coefficient (pc). There are some indications for interindividual differences in this pc. Determinants of this variation are not known, but an obvious factor might be the blood lipids content, related to the nutrition status of the study subject. For collection of end-exhaled air, equipment such as the BIOVOC® container can be used and gave good estimates of toluene venous blood levels (K. Jones, Buxton). For organic solvents with fast kinetics, a breath sample must not be collected right after the end of exposure, but a good compromise is to take the sample 10 min after cessation of exposure in a clean environment. For solvents with a longer half-life such as chlorinated hydrocarbons, the moment of sample collection is less critical and is recommended at the end of a work week because of possible bioaccumulation of the solvent in the body. Toxicokinetic models are useful for interpretation of results. The use of these models showed that it might be difficult to relate alveolar air concentrations to preceding uptake if exposure was intermittent and peak exposures occurred (keynote by Dr C. Viau, Montreal, claude.viau@umontreal.ca). Further laboratory studies will be needed to understand the patterns of excreted substances (and their metabolites) after peak exposures (Dr J. Bessems, Zeist, bessems@chemie.tno.nl). These approaches also provide information on interindividual variability, which is important in the setting of short-term exposure limits (STELs). Since many organic solvents have a deleterious effect on the brain, it would be interesting to know how well alveolar air levels reflect arterial blood levels. Such questions will not be answered in volunteer studies simply because it is difficult to find those volunteers.

A special field is the use of exhaled breath condensate (EBC) for those substances with a lower volatility and good solubility in water. Merits and limitations of the use of EBC were addressed in a keynote lecture by Dr Mutti (Parma). It offers an attractive non-invasive technique to obtain information on the toxicology and pathology of the airways. Metals are bound to carriers in aerosols and can thus be retrieved with EBC. An interesting hypothesis that can be investigated using EBC is the depletion of glutathione (GSH) by toxicants that thereby disturb this important protective mechanism against the deleterious effects of strong oxidants. Especially co-exposure to heavy metals and transition elements could increase the risk of oxidative damage, due to the enhanced formation of hydroxyl radicals. A number of markers of effect can

be measured in EBC, including pH, interleukins, malondialdehyde and hydrogen peroxide. There is a need to standardize collection of EBC. Equipment for collection of EBC in field studies using a portable device and disposable tubes is now available (c.valesi@libero.it).

More invasive methods

Methods for blood collection are perhaps not the most preferred medium of the study subject but is definitely a very interesting medium for a toxicologist. Nevertheless, for some substances it can be very important to obtain a blood value. The lead blood content has been used for many decades to monitor occupational and environmental exposures for regulatory purposes, but also to investigate possible neurotoxic effects in epidemiological studies. A field currently in rapid progression is the determination of peripheral blood protein adducts. During the meeting, new or improved methods were presented for haemoglobin adducts of 2,4- and 2,6-TDI, N,N-dimethylformamide (DMF), acrylamide, acrylonitrile, 1-bromopropane, and 1,3-butadiene, and serum albumin adducts of 1,2-epoxy-3,4-butanediol. To establish a method takes many years of research. First, the metabolic pathway must be known to identify an adduct that reflects activity of the ultimate toxic intermediate. Second, a stable adduct must be chemically characterized, usually by testing labelled substances *in vitro* or in experimental animals. To obtain an analytical method for quantification, it is necessary to synthesize standards and also (if possible, deuterated) internal standards. Usually methods are validated in animal experiments and then in humans. Because most substances studied are (suspected) carcinogens, it is not possible to perform volunteer studies, so methods have to be tested on field samples. Most methods are focused on specific neutrophylic targets such as N-terminal valine.

A more comprehensive approach to study protein adducts uses techniques commonly available in proteomics research such as LC-ESI/MS/MS by Dr Lindh (Lund, christian.lindh@ymed.lu.se). Binding of trihydroxybutyl adducts with human serum albumin (HSA) was studied *in vitro* by incubation of 1,2-epoxy-3,4-butadienediol (EBD) with HSA. A tryptic digest of the conjugates was analysed using a nanospray-hybrid quadrupole time-of-flight MS (QTOF) to characterize specific binding sites of EBD to HSA. Theoretical predictions of MS/MS spectra were used to identify possible adducts from the enzymatic digest. EBD bound primarily to Lys and His, also to Glu and N-terminal Asp, but not to Cys (presumably because of steric hindrance). Of the 19 characterized adducts, six were selected as potential biomarkers. The development of analytical methods using LC MS/MS for quantification of these adducts is currently in progress.

N,N-dimethylformamide (DMF) is used as a solvent in the plastic manufacturing industry. It is readily absorbed by the skin. In a 3-month field study, levels of airborne DMF, N-methylcarbamoylvaline adduct in globin, and urinary metabolites (N-methylformamide and N-acetyl-*S*-(N-methylcarbamoyl)cysteine) were determined (Dr Mráz, Prague). The N-methyl-carbamoyl adduct at the N-terminal valine of globin is a metabolic product of DMF with a lifetime of 4 months in the body (as other globin adducts). It is determined after conversion to 3-methyl-5-isopropylhydantoin (MVH). Analyses were carried out using GC-MS/MS. In total, 52 workers in five different plants were recruited for this study. Results showed a clear association between the level of MVH and other measured parameters, with the

restriction that parameters must be integrated as a mean value throughout the 3 months. In another study of the same group, the presence of a new adduct in humans, namely an N-methylcarbamoyl-lysine adduct in globin, was demonstrated. Two methods of globin analysis were performed: Edman degradation to convert the valine adduct to 3-methyl-5-isopropylhydantoin (MVH) and enzymatic hydrolysis with protease to release free N-methyl-carbamoylvaline (MVU) and N_e-(N-methylcarbamoyl)lysine (MLU). All three adducts could be detected. MLU correlated significantly with MVH levels.

Isocyanates and thermal degradation of plastics is still a hot topic

Fumes from heated plastics contain isocyanates that may cause occupational asthma. Dr Mráz (Prague) presented studies of his group and of the group of Dr Sakai[2] (Japan) on the development of specific adducts of 2,4- and 2,6-toluene-diisocyanate with globin. Both isocyanates bind at the N-terminal valine of globin. The adducts can be hydrolysed by Edman degradation as isomeric 3-(aminotolyl)-5-isopropylhydantoins ('toluenediamine-valine-hydantoins', TVHs), namely 2-TVH, 4-TVH (from 2,4-TDI) and 6-TVH (from 2,6-TDI). Reversed phase LC/MS was used for analysis. In workers exposed to TDI, 6-TVH could be detected (range 1.3–73.9 ng g^{-1} globin), while the 4-TVH level was below the limit of determination (1 ng g^{-1} globin). The level of 6-TVH correlated with the urinary level of 2,6-toluenediamine. In an animal study, rats were exposed to 2,4-TDI vapour (0.46–1.87 mg m^{-3}) for 4 h. Levels of 4-TVH, total (free + conjugated) 2,4-toluenediamine in urine (2,4-TDA-U) and total 2,4-TDA in plasma (2,4-TDA-P) were determined. It was shown that 4-TVH levels correlated best with the 2,4-TDI uptake. Finally, progress was made in the development of biomonitoring of 4,4′-methylenediphenyl diisocyanate (MDI; Morita, Tokyo, xvh@msa.biglobe.ne.jp). In workers engaged in tunnel construction, serum 4,4′-methylenedianiline (MDA) levels were determined after derivatization with heptafluorobutyric acid by GS/MS with negative chemical ionization. The serum MDA concentrations in three workers decreased with a half-life of 17–42 days.

Aggressive graffiti remover

N-methyl-pyrrolidone (NMP) is an extremely stable solvent with a low vapour pressure and a broad solubility spectrum being miscible with water and a variety of organic solvents. It is used as a formulating agent for the production of pesticides and dyes, in the production of catalysts, as a cleaning and paint-stripping agent, and as a graffiti remover. It has been reported to be toxic to unborn rats; is a possible carcinogen in mice and rats; and causes headache and irritation of the eyes, skin and respiratory system in humans. The human body readily absorbs NMP in the gastrointestinal tract, in the airways and through the skin. In a toxicokinetic study with human volunteers, the main metabolite excreted was 5-hydroxy-N-methyl-2-pyrrolidone (5-HNMP, 60–65% of the administered dose; Dr Carnerup, Lund, martin.carnerup@ymed.lu.se). Other metabolites were 2-hydroxy-N-methylsuccinimide (35–40% of the dose) and N-methyl-succinimide (0.1%). One to 2% of the dose were excreted as the parent compound. Interestingly, the difference in total excretion of NMP and its metabolites seemed higher after exposure to NMP in humid air than in dry air. Furthermore, uptake by inhalation alone cannot explain the amounts of

NMP and its metabolites recovered in urine, even after exposure in dry air. This indicates that the dermal route is important. The latter fact was confirmed in another study where urine of workers exposed to NMP during graffiti and paint removal was analysed for 5-HNMP (Dr Cocker, Buxton, john.cocker@hsl.gov.uk). Airborne NMP concentrations were low, both in graffiti removal and in paint removal by dipping. Skin exposure was significant for the hands when wiping (14–550 $\mu g\,cm^{-2}\,min^{-1}$) and dipping 0.001–250 ($\mu g\,cm^{-2}\,min^{-1}$). All graffiti removal workers had detectable levels of 5-HNMP in their urine. Manual dipping resulted in higher levels of urinary excreted metabolites than semi-automated dipping. Finally, in a study where workers were monitored during paint stripping of furniture, it was concluded that airborne concentrations of NMP were low, 1.2% of the occupational exposure level (OEL). The maximum excretion of 5-HNMP corresponded to 5.4% of the value expected for inhalation exposure at the level of the OEL.

Phthalates and scoubidous

Considerable efforts have been undertaken to improve analytical methods to estimate internal uptake of phthalates. These substances are additives to plastics, so-called plasticizers, e.g. in PVC threads used by (young) children in a revival of macramé knitting (called Scoubidou®). This hype refuelled the discussion on the use of these plasticizers in children's toys, in the media during last summer in The Netherlands and Germany.[3] These additives are known liver peroxisome proliferators, are nephrotoxic and suspected tumour promoters and epigenetic carcinogens causing Leydig cell tumours, pancreas tumours and mononuclear leukaemia's in experimental animals. Furthermore, these products may have adverse effects on fertility and offspring. Exploratory work was done by H. Koch and co-workers in Erlangen (holger.m.koch@ipasum.uni-erlangen.de) to study the complex biotransformation routes of di(2-ethylhexyl)phthalate (DEHP) in humans. A volunteer had deuterium-labelled DEHP for breakfast (0.35, 2.2 and 48 mg on a sandwich on three separate occasions). 5-Carboxy-MEHP, 5-hydroxy and 5-oxo-MEHP were the primary urinary metabolites during the first 8 h of excretion, with half-lives of 5, 10 and 15 h, respectively. Twenty-four hours after administration, 67% of the dose was recovered in urine, comprising 23% 5-OH-MEHP, 23% 5-carboxy-MEHP, 15% 5-oxo-MEHP and 6% MEHP. This finding is not in line with previous reports of MEHP as a major metabolite in humans and other reports suggesting that a majority of the dose is excreted in the faeces. It is suggested that the oxygenated MEHP metabolites may be more relevant because they are more toxic than parent MEHP. For recent exposure to DEHP, 5-OH-MEHP with an excretion half-life of 12 h appears to be the most suitable biomarker. 5-Oxo-MEHP, 5-carboxy-MEHP and 2-carboxy-MEHP are more useful for determination at steady-state because they have a substantially longer excretion half-life. In a discussion following the presentation of these interesting new results, it is suggested that more human subjects are studied before final conclusions are drawn. The most recent finding[4] of similar metabolite patterns in subjects in the general population confirms the finding of the $n=1$ volunteer study (for further information contact, Dr Angerer at nicole.gaidischki@ipasum.imed.uni-erlangen.de).

As part of the European Union project Biomonitoring of Carcinogenic Substances (BIOMONECS), Dr Rettenmeier (Essen, a.w.rettenmeier@uni-essen.de) synthesized standards for 6-OH-MEHP, 5-carboxy-MPP and for 5-OH-MEHP and 5-oxo-

MEHP, including some deuterated analogues used in the validation of a method developed by Hoppe and co-workers (Bremen, hans-wolfgang.hoppe@mlhb.de) to measure these metabolites on a GC-MS/MS system (triple quadrupole instrument) in negative chemical ionization mode (upon derivatization with pentafluorobenzylbromide and trimethylsilyltrifluoroacetamide). A limit of quantification was achieved of 2.3–2.5 $\mu g\,l^{-1}$ urine. This new method was compared with the LC-MS/MS-based approach used by the group in Erlangen by exchange of samples from 43 human subjects. There was a remarkable high correlation ($R^2 = 0.9969$ for MEHP, 0.9954 for 5-OH-MEHP, and 0.9905 for 5-oxo-MEHP), giving confidence that the methods are reliable and can be used to quantify background levels of secondary DEHP metabolites in the general population. Background values in six European Union countries measured during the BIOMONECS project were 10.8 $\mu g\,g^{-1}$ creatinine for MEHP, 37.2 $\mu g\,g^{-1}$ for 5-OH-MEHP, 27.5 $\mu g\,g^{-1}$ for 5-oxo-MEHP, and 54.3 $\mu g\,g^{-1}$ for 5-carboxy-MPP.

Acrylamide and potato chips

Acrylamide (AA) is a well known neurotoxin and confirmed rodent carcinogen (IARC classification '2A') present in, for example, potato chips, toasted wheat products, drip grind coffee and tobacco smoke. The daily uptake from the diet is estimated to be 25–100 μg. AA is also used in industry for the production of polyacrylamides, as a grouting agent in tunnel construction and for the production of fire-protective glass. In the body, acrylamide is metabolized by CYP 2E1 to glycidamide. In a study performed at the University of Erlangen-Nuremberg (T. Schettgen and co-workers, thomas.schettgen@ipasum.uni-erlangen.de) a method was developed to determine N-terminal valine haemoglobin adducts of acrylamide (N-2-carbamoylethylvaline, AAV) and its metabolite glycidamide (N-(*R*,*S*)-2-hydroxy-2-carbamoly-ethylvaline, GAV). The adducts were analysed using N-alkyl Edman degradation and the derivatives were stabilized using H_2SO_4 and acetone. Dipeptide standards were used for calibration. The adducts were analysed on a GC-NCI-MS system using a rather laborious procedure (over 3 days). Adduct levels were determined in a group of 17 workers active in the production of fire-protection glass, in smoking reference subjects ($n = 16$) and in non-smoking reference subjects ($n = 13$). Median adduct levels were 215, 83, and 18 pmol AAV g^{-1} globin, and 121, 44 and 18 pmol GAV g^{-1} globin.

In a second study, it was attempted to develop methods for the determination of mercapturic acids (MA) of acrylamide (N-acetyl-*S*-(2-carbamoylethyl)-L-cysteine, AA-MA) and glycidamide (N-(*R*/*S*)-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine, GA-MA). In non-smokers, the median urinary metabolite level (range) for AA-MA and GA-MA was 29 (3–83) $\mu g\,l^{-1}$ and 5 (< LOD–14) $\mu g\,l^{-1}$. For smokers these values were 127 (17–338) and 19 (3–45) for AA-MA and GA-MA, respectively. The correlation coefficients of the association between mercapturic acids and haemoglobin adducts were 0.783 and 0.823 for AA-MA/AAV and GA-MA/GAV, respectively. Because of the different half-lives of these products in the body, it must be assumed that exposure reached a steady-state in the studied subjects. GA-MA was first reported in humans in this study. The use of these biomarkers of exposure in epidemiological studies may help to study the suspected carcinogenic potency in humans.

In a study by K. Jones and co-workers (Buxton) on the paper and pulp industry, 60 workers exposed to acrylamide supplied two blood samples for the determination of AAV (the method was similar to that described above) with a 3-month interval. Information on inhalation exposure was based on (at least) two full shifts during that period. For the first time a correlation between haemoglobin adduct levels and airborne exposure for acrylamide was demonstrated. Such a correlation indicates that exposure must have been fairly constant over the period studied. From this correlation it was deducted that the current maximum exposure limit of 0.3 mg m^{-3} in the UK corresponded to an adduct level of 1.5 nmol g^{-1} globin.

1-OHP still going strong as a biomarker for PAH exposures

Biological monitoring of polycyclic aromatic hydrocarbons (PAH) was well covered in nine contributions. 1-Hydroxypyrene (1-OHP) is still the most used biomarker to evaluate exposures in occupational groups but also in the general population. The big issue is how to interpret the urinary excretion of 1-OHP in terms of exposure to carcinogenic PAH which are mostly less volatile that pyrene. A way to address this problem is to determine a so-called PAH profile in the industrial product (e.g. in creosote oil), skin contamination samples (dermal exposure) or in airborne gaseous/particle phases (inhalation exposure): a selection of PAH covering a wide range of different congeners with different physical chemical properties (such as the selection of 16 PAH by US/EPA). A field test of the use of the sum of 1- and 2-naphthol (metabolites of naphthalene, classified by IARC as possibly carcinogenic to humans, group 2B), showed that this biomarker can be used if exposures to naphthalene (exceeding 40 μg m^{-3}) are greater than the uptake by tobacco smoking (R. Preuss, Erlangen, ralf.preuss@ipasum.uni-erlangen.de). Methods were recently published by Preuss and Angerer (2004). 1-OHP and four isomeric hydroxyphenanthrene (HPh) metabolites were measured in urine of 255 male workers exposed during production of coke, graphite electrodes and refractories (B. Rossbach, Mainz, rossbach@uni-mainz.de). A significant correlation was found between excretion of 1-OHP and the HPh metabolites. Smoking was a minor confounder in this group. It was concluded that HPh metabolites could be complementary, but not an alternative to 1-OHP: 1-OHP is a marker more specific for particle bound PAH whereas HPh-metabolites are associated with more volatile PAH.

A high (dermal) uptake of PAH was observed in Finnish soil remediation workers as indicated by the urinary excretion of 1- and 2-naphthol, 1-, 2-, 3-, 4- and 9-phenanthrol, and 1-OHP in pre- and post-shift samples (Elovaara et al. 2003) (E. Elovaara, Helsinki, eivor.elovaara@ttl.fi). Low concentrations of 1-OHP were found in workers at a vulcanization department of a rubber industry in Sweden (B. Jönsson, Lund, bo.jonsson@ymed.lu.se).

A promising alternative for 1-OHP is the determination of 3-hydroxybenzo[a]pyrene (3-HBP, C. Champmartin, Vandoeuvre, catherine.champmartin@inrs.fr). In 27 smokers, matched to non-smokers by occupation and socio-economic class, median 3-HBP values were 0.023 nmol mol^{-1} creatinine for smokers and 0.011 nmol mol^{-1} creatinine for non-smokers, based on 24 h urine samples (all voids from one 24 h period pooled into a single sample). For 1-OHP, these values were 0.115 and 0.032 μmol mol^{-1} creatinine, respectively, showing a moderate influence of smoking. Conventional reversed-phase HPLC with fluorescence detection is not

sufficiently sensitive to detect 3-HBP. Instead, a column switching HPLC system should be used with a limit of detection of 0.05 ng l^{-1} urine.

The ISBM meeting was combined with the meeting of the Biological Exposure Index (BEI) Committee of the American Conference of Governmental Industrial Hygienists (ACGIH). One of the highlights of that meeting was the agreement on a BEI for occupational exposure to PAH, based on the determination of 1-OHP. This will certainly stimulate further use of 1-OHP, although other metabolites offer suitable alternatives for exposure monitoring.

More new urine-based methods

Several new methods were presented. Dr Cocker and co-workers (Buxton, john.cocker@hsl.uk.gov) developed a method to detect *m*-xylylene diamine (mXDA) in urine. mXDA is a cross-linking agent used as an epoxy resin hardener. Its main health effect is irritancy and it is a possible skin sensitizer. The method was developed to check the effectiveness of personal protective equipment of workers lining containers with an epoxy resin. The method is comparable with already existing methods for aromatic amines and is based on hydrolysis of the urine sample, followed by extraction into diethylether, evaporation and derivatization with heptafluorobutyric anhydride and analysis by GC/MS. The sensitivity is comparable with aromatic amines and the stability of urine-mXDA is at least 7 days. Background levels seem not to be a confounding factor because in ten non-exposed humans no mXDA could be detected. The suitability of the method was demonstrated by the study in the container plant where exposed workers showed significantly higher mean post-shift urinary values as compared with pre-shift values.

Metabolites of pyrethrins and pyrethroids can be detected in urine (Dr Leng, Leverkusen, gabriele.leng.gl@bayerindustry.de). Pyrethrins are among the insecticides most often used. Mixed exposure to pyrethrins and pyrethroids is common. With sophisticated HRGC/MS equipment, it is possible to detect both metabolites of synthetic pyrethroids (*cis*-DCCA, *trans*-DCCA, *cis*-DBCA, 3-PBA and FPBA) and pyrethrins (chrysanthemumdicarboxylic acid, *trans*-CDCA) in one urine sample. Spraying pyrethrum by workers resulted in *trans*-CDCA levels of 0.3–1 μg l^{-1} urine. Dermal exposure of 30 volunteers resulted in background levels of *trans*-CDCA lower than 0.1 μg l^{-1}. The highest concentrations (up to 14 μg l^{-1}) were observed immediately after exposure, with values returning to background (<0.1 μg l^{-1}) after 5 days. Following an oral exposure, *trans*-CDCA was eliminated during the first 48 h. In most of the subjects, a background level of 3-PBA as well as of *cis*/*trans*-DCCA was found. The overall conclusion is that urine *trans*-CDCA is a reliable biomarker of pyrethrin exposure within 48 h after exposure.

For the detection of aromatic amines in the rubber industry, several methods are available: monitoring of aniline (AN) and *o*-toluidine (OT) in urine and monitoring of haemoglobin adducts to AN, OT, 4-aminodiphenyl (4-ADP), and 2-napthylamine (2-NA). 4-ADP and 2-NA are potential contaminants of industrial chemicals. Aromatic amines may also be released during the production process when using vulcanization accelerators and stabilizers such as diphenylguanidine and di-*o*-tolylguanidine. The latter metabolite reflects exposure from the past few months. In this study presented by Dr T. Weiss (tobias.weiss@ipasum.imed.uni-erlangen.de), 69 workers from four rubber production plants were monitored. Air concentrations of

AN and OT were below the OEL. Nevertheless, exposed workers appeared to have higher AN and OT urine levels than the general population. A similar pattern was observed for adduct levels of AN and OT. Differences between departments were apparent, the curing department having the highest and the mixing department the lowest levels. In the curing department, the highest ambient levels are expected because of the vulcanization process. No adducts of 4-ADP and 2-NA were detected. The correlation between air and urine levels was not high. Skin exposure is a likely confounding factor.

Relevance of susceptibility markers

Interindividual differences in susceptibility are beginning to have impacts on regulatory authorities. The OEL for dichloromethane established by the ACGIH was reduced to protect individuals who are lacking an active glutathion-*S*-transferase (GST) T1. The availability of knock out mice derived from C57B6, with a lacking activity of cytochrome P-450 (CYP) 1A1 or 1A2, or aryl hydrocarbon (AH)-receptor triggered some research trying to understand the role of different CYP isoenzymes in the metabolic activation of benzo[a]pyrene (BP) and 4-aminobiphenyl (4-ABP). Knocking out CYP1A2 had no effect on DNA–adduct levels in urinary bladder and liver following exposure to 4-aminobiphenyl (4-ABP) and reduced BP–DNA adduct levels two-fold (G. Talaska, Cincinnati, glenn.talaska@uc.edu). Knocking out the AH-receptor resulted in a 90% reduction in BP–DNA adducts. It is suggested that PAH rely on metabolic activation of different CYP isoenzymes. Knocking out one enzyme is apparently compensated for by an increasing contribution of other isoenzymes of the CYP family. This might explain no more than moderate effects of 1.2–1.5 risk ratio for formation of PAH–DNA adducts, observed so far in epidemiology studies (Vineis et al. 2001).

Molecular markers for susceptibility to neurotoxic substances were discussed in a keynote presentation by L. G. Costa (Seattle, lgcosta@u.washington.edu). Paraoxonase 1 (PON1) is a member of the gene family that also comprises PON2 and PON3, all aligned next to each other on the long arm of chromosome 7. PON1 is involved in the metabolism of certain organophosphorus (OP) insecticides (e.g. chlorpyrifos oxon, diazoxon — the active metabolites of chlorpyrifos and diazinon) and nerve agents (e.g. sarin), as well in the metabolism of oxidized lipids and of certain drugs (Costa et al. 2003). PON1 is expressed in liver and in blood, where it is tightly bound to high-density lipoproteins (HDL). Several polymorphisms have been identified in PON1: a Glu to Arg substitution at position 192 affects the enzyme's catalytic ability toward different substrates, while a T/C substitution at position –108 affects PON1 levels of expression. In epidemiological studies, it is important to consider PON1 'status', rather than simply genotypes, as the former takes into account all important variables, i.e. genotype and activity. This is accomplished with an enzyme assay, involving two PON1 substrates (paraoxon and diazoxon), that provides a functional assessment of the plasma PON1 192 alloform as well as the plasma levels of PON1 for each individual.

The role of PON1 in determining susceptibility to OP insecticides has been studied in transgenic animal models. PON1 knockout mice are exquisitely sensitive to the acute toxicity of chlorpyrifos oxon and diazoxon, but surprisingly, not of paraoxon, the substrate after which the enzyme was named. This is due to a very low catalytic

efficiency of PON1 for paraoxon. Experiments in 'humanized' transgenic mice (expressing either the human Q192 or the R192 PON1 alloform on a knockout background) indicated that hPON1 R192 animals were more resistant to chlorpyrifos oxon toxicity than hPON1 Q192 mice. PON1 activity is low in newborn animals and humans, suggesting a possible role in the age-dependent susceptibility to OP toxicity. In a study carried out in PON1 knockout mice it was found that exposure to chlorpyrifos oxon on postnatal days 4–21 caused significant neurotoxicity. Interestingly, *in utero* exposure to chlorpyrifos has been shown to cause microcephaly, often associated to mental retardation, in children born to mothers with low PON1 activity (Berkowitz et al. 2004).

Growing interest for biological monitoring in the European Union

The perspectives for the future of the use of biomonitoring are good because the fundaments for applications of biological monitoring for health risk assessment are laid down in European Union Directives (95/320/EC). The Scientific Committee on Occupational Exposure Limits (SCOEL) appointed by the European Commission in 1995 to establish occupational exposure limits (OELs), has the possibility to introduce so-called biological limit values (BLVs) for priority substances (H. Bolt, Dortmund, bolt@ifado.de). At present, there are BLVs for lead in blood (30 μg/100 ml blood) and carbon monoxide (4% carboxyhaemoglobin in blood). Suggestions for other BLVs have been made for both 2-butoxylethanol (1996) and sulfotep (1997). Before the end of 2004, SCOEL is expected to adopt a definition for BLVs and extend/revise chapter 11 'Health-based biological limit values' of a report *Methodology for Derivation of OELs* (1997). SCOEL also has the possibility to assign skin notation to chemical substances that are substantially skin absorbed (relative to uptake by inhalation and through the gastrointestinal tract). For exposure assessment of these substances, biological monitoring is the preferred approach.

The field of research and routine laboratories appears well prepared to accommodate the use of biological monitoring. There are external quality assessment schemes in Finland, Germany, Canada and the USA (Schaller, Erlangen KH.Schaller@rzmail.uni-erlangen.de). The German scheme is the most comprehensive, covering 76 biomarkers relevant for occupational and 38 for environmental health (covering in total 80 chemical substances), with 350 laboratories participating. The overall median success rates (range) for the participating laboratories was 70 (58–87)%. Clinical chemical laboratories and routine laboratories showed the best analytical performance while some governmental laboratories had the lowest success rates. The European Union currently supports the transfer of established and new methods for carcinogenic substances from university research laboratories to private routine laboratories (BIOMONECS). Hopefully this will lead to a reliable European-wide infrastructure that can supply a routine service for the users of biological monitoring.

Acknowledgements

The authors thank the cited contributors for consent to publish this report and for suggestions made to improve its quality.

Notes

1 Proceedings of the meeting will be published in *Toxicology Letters* (edited by Dr G. Triebig. E-mail: GTriebig@med.uni-heidelberg.de).
2 Dr Sakai died during the summer of 2004. His co-workers can be reached at opc@msa.bibglobe.ne.jp
3 After the conference, the European Commission announced a complete ban on the use of certain phthalates in plastics in toys such as the colourful Scoubidou® PVC threads to be effective in 2 years.
4 This was brought to the attention of the authors after the meeting by Dr Angerer.

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