Comparison of Metabolic Stability and Metabolite Identification of 55 ECVAM/ ICCVAM Validation Compounds between Human and Rat Liver Homogenates and Microsomes – a preliminary Analysis*

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Summary

In vitro methods to produce metabolic information have increasingly been applied in toxicity risk assessment. In the current contract project of JRC/ECVAM In vitro-Toxicology Unit, 55 organic chemicals, mostly drugs and pesticides, most belonging to ECVAM/ICCVAM validation compounds, expected to be analyzable by LC-MS technique, were subjected to a feasibility study. The simple experimental setup consisted of one concentration of a chemical (25 µM), enzyme preparation (human or rat liver homogenate or microsomes), a set of cofactors (NADPH, UDPGA, PAPS, GSH), 4 time points (0, 15, 30, 60 min, including cofactor-less tubes). Metabolites produced were analyzed and tentatively identified by LC-MS techniques.

Most of the chemicals were metabolized and metabolites were tentatively identified by TOF-MS analysis. For some chemicals, about 10 or even more metabolites were detectable (e.g. thioridazine, verapamil, amitriptyline). Altogether 11 out of 55 did not display any metabolites under the experimental conditions of this study. Regarding the metabolites formed, there were mostly quantitative differences, but about 20 substances displayed also species-dependent qualitative differences, i.e. a major metabolite was formed in one species, but not in the other. For most chemicals, differences between microsomes and homogenates were relatively modest at least in the initial analysis.

The results demonstrate that LC-MS approach is feasible and rather efficient in providing useful metabolic data from a simple experimental setup. More complex analyses, e.g. quantitative assessment of differences between species or biological preparations, or in vitro-in vivo extrapolations, require more complex approaches and a collection of appropriate, preferably curated, data bases of in vivo characteristics of the studied chemicals.

Keywords: metabolic stability, metabolite formation, ECVAM/ICCVAM, validation compounds, liver preparations, human, rat, LC-MS techniques

1 Introduction

Elucidation of metabolic stability and metabolic routes is important in early phases of drug development, because metabolism determines to a great extent the pharmacokinetic properties of most drugs and is often behind interactions, metabolic idiosyncrasies and so on (Boobis et al 1999; Pelkonen et al 2001; Pelkonen 2002). Likewise, information on metabolic properties of any chemical is a significant piece in toxicological risk assessment. Actually *in vitro* methods to produce metabolic information have increasingly been applied also in chemical risk assessment (Coecke et al 2006).

Human-liver derived *in vitro* systems have proved useful in early studies of metabolic stability, in identifying enzymes capable of metabolising, and interacting with, drugs in use or under development and several of these systems are currently

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under validation as to their predictive power to the *in vivo* situation (for reviews see e.g. Pelkonen et al 2009; Pelkonen et al 2002; 2005; Pelkonen and Raunio 2005).

In the current project contract with ECVAM, 55 organic chemicals, mostly drugs and pesticides, 51 of them belonging to the set of the ECVAM/ICCVAM validation compounds, was selected for *in vitro* metabolism studies. The goals of the investigations described in this paper were to investigate whether a relatively simple and not-too costly experimental setup were feasible and would provide appropriate data to characterise, with human and rat liver homogenate or microsomal preparations, the metabolic stability (i.e. disappearance of the parent substance from incubations with human liver preparations and appropriate cofactors for metabolism) and the appearance of metabolic products. With this approach we hope to develop, in the end, a robust and reliable testing system to obtain useful *in vitro* information to predict the pharmacokinetic behaviour of organic chemicals in the *in vivo* situation. Equally important is to assess similarities and differences between homogenate and microsomes, as well as between human and rat. The basic approach is expected to be suitable for the characterization of metabolic capability of any tissues or cell cultures from various animals or humans. Actually an analogous approach has been widely employed by us for several years in research and contract services.

2 Materials and methods

Chemicals
Most chemicals were supplied by ECVAM. The data required before studies were molecular weight and solubility into solvents which are compatible with the conditions in *in vitro* incubations with human liver preparations. Other chemicals were obtained mainly from Sigma Chemical Company (St. Louis, Missouri, USA) and Boehringer (Ingelheim, Germany) and were of the highest purity available.

Human liver samples
Human liver samples used for these studies have been obtained from kidney transplantation donors. The Ethics Committee of the Medical Faculty of the University has approved the collection of surplus human tissues for research and service purposes. Liver preparations have been extensively characterised to be used for the primary screening (sufficient model activities, no known polymorphisms, expected effects of model inhibitors). The present studies will be performed with a pool of 5 to 7 active and thoroughly characterised liver preparations.

Preparation of liver homogenates and microsomes
Normal-looking tissue was homogenised in four volumes of ice-cold phosphate buffer, pH 7.4, and the resulting homogenate was used as such. Microsomes were separated by differential centrifugation. Briefly, homogenate was first centrifuged at 10,000 x g for 15 min and the supernatant was then centrifuged at 100,000 x g for 60 min. The final microsomal pellet was suspended in 0.1 M phosphate buffer.

Tissue samples from rats
Liver samples from Rat (Sprague-Dawley and Wistar strains, originally used for other purposes) stored in the deep freeze at -80°C were used for studies. Basically the same approach was used for liver tissue samples of rat as for human, although the recovery and purity of the subcellular organelle were rigorously checked in the case of human.

Incubation of a chemical
The studied compounds were incubated with human and rat liver preparations (microsomes and homogenate) in the presence of appropriate cofactors. Asingle analytically adequate concentration (25 μM) was used. In the microsome incubations, NADPH (for producing oxidized metabolites) and UDP-glucuronic acid (for producing glucuronic acid conjugates) were added, and in the homogenate incubations also GSH (for producing glutathione conjugates), PAPS (for producing sulphate conjugates) were added. Samples were taken from incubates at 0, 15, 30, and 60 min and treated with ice-cold acetonitrile to stop the biological reactions. An incubation without cofactors was conducted in parallel and one sample was taken (60 min) as control. Additionally, for each set of study compounds, a blank incubations for each species and each preparation (cofactors but no study chemicals) were conducted. All incubations except blanks were performed in duplicate.

Analysis of the samples by liquid chromatography mass spectrometry
A Waters Alliance 2695 high performance liquid chromatographic system (Waters Corp., Milford, USA with autosampler, column oven and vacuum degasser was used with Phenomenex Luna-C18, Waters XTerra RP18, Waters XTerra MS C18, Waters XBridge Shield RP18 and Waters XBridge C18 columns (all with 2.0/2.1× 50 mm dimensions and 3-3.5 μm particle size). Phenomenex Luna C18 (2×4 mm) precolumn was used with all columns. Temperature of column oven was 35ºC and injection volumes used were 10-20 μl. The flow was split post-column with an Acurate Post-Column Stream Splitter (LC Packings, Amsterdam, The Netherlands) with a ratio of 1:3 to MS and waste, respectively. The data was acquired with a Micromass LCT time-of-flight (TOF) mass spectrometer (Micromass Ltd., Manchester, England) equipped with a LockSpray electrospray ionization source, used both in positive and negative ion polarity. Cone voltages between 16-50 V were used (optimized for each compound). Capillary voltages of 3.5 kV and 3.0 kV were used at positive and negative ion mode, respectively. Nitrogen was used as a nebulizer and drying gas with flow rates of 100 l/h and 800 l/h, respectively. The source and desolvation temperatures used were 150ºC and 240ºC, respectively. Leucine encephalin ($[M+H]^+$ m/z 556.2771) and raffinose ($[M–H]^–$ m/z 503.1612) were used as a lock mass compounds in accurate mass measurement with positive and negative ion modes, respectively, and were delivered into LockSpray probe with syringe pump to obtain about 160 counts/s. The mass spectrometer and HPLC system were operated under Masslynx 4.0 software.

Chromatographic conditions and method development
In method development the study compounds were devided into batches of 3-10 compounds, based on their chemical struc-

tures or acid/base properties, and LC/MS conditions for each compound were briefly optimised by acquiring test data for all compounds in a single batch at the same time. In the beginning of method development, some runs with varying gradient strength (slope of the acetonitrile or methanol proportion) were acquired, followed by runs with various aqueous phase pH conditions between 2.4 and 9.8 and different MS ionisation polarities to optimise the ionisation efficiency (i.e. the detection sensitivity). For compounds with poor peak shape, also the effect of different columns (see above) were tested for improving chromatographic peak shapes.

Sample preparation
The incubation samples were thawed at room temperature (RT), shaken and centrifuged for 15 min at 16,100 × g (Eppendorf 5415D, Eppendorf AG, Hamburg, Germany) and pipetted to Maximum recovery vials (Waters Corporation, Milford, Massachusetts, USA) to wait for an autosampler run.

Data processing for metabolic stability and identification of metabolic transformations
The incubations were analysed with LC/PDA/TOF-MS system as described above. For the compounds having UV activity, the PDA response in selected wavelength was used for metabolic stability analyses. For non-UV active compounds, the TOF-MS data with ^{13}C isotope ions (to avoid detector saturation) was used for metabolic stability analyses. The average of duplicate incubations was used as peak area for metabolic stability calculations.

The metabolites were mined from the acquired data by comparing the LC/TOF-MS data from metabolized sample with the negative control (blank sample), both manually and by using the MetaboLynx software. Accurate mass data was used to obtain elemental composition for all found metabolites to identify the respective biotransformations. In addition, the in-source fragment ion data obtained for some compounds were used to further elucidate the metabolite structures.

Metabolite profile of a chemical
To generate the metabolite profile for each study compound in each liver preparation, the LC/TOF-MS peak areas of each found metabolite were measured and a column chart was created for each sample. Based on the relative abundance of different metabolites and their tentative structures, proposals of biotransformation pathways were drawn, and the results of a chemical in different species and liver preparation were compared. It has to be stressed that, absolute identification and quantification was not possible, and instead, metabolite "fingerprints" were produced and compared.

3 Results and discussion

3.1 Development of analytical methods

LC/TOF-MS methods were developed with parent compounds by optimizing chromatographic behaviour (retention and peak shape) and mass spectrometric ionisation. Several reverse-phase HPLC columns and eluents at pH range 2-10 were used during these processes, and conditions giving the best performance for each substrate were used. For some of the compounds the analytical methods did not work well due to the poor retention and/or poor mass spectrometric ionisation properties of the compounds, and for these also HILIC chromatography was tested.

LC/TOF-MS methods used were well suitable for most of the 55 substances. With 4 substances, the LC/MS-MS methods had to be used due to poor specificity or sensitivity of the LC/TOF-MS approach. Suitability of LC/MS for most analyses was naturally expected, because the pre-selection of compounds from the list of all ECVAM/ICCVAM validation compounds was mostly done on the basis of analytical feasibility. The most notable exceptions were compounds with a large number of halogen substituents, relatively small molecules and those substances with weak ionization properties.

A detailed and comprehensive description of analytical methods for each studied substance is under preparation and will be published in due course.

3.2 Comprehensive categorical survey of results

We performed an initial, principally categorical survey and comparison of all the results obtained. These assessments are collected in Table 1.

Tab. 1: Comprehensive categorical survey of the results

		Apparent disappearance[1]		Metabolites formed[2]	
Compound	Preparation	Human	Rat	Human	Rat
Carbamazepine	Homogenate	very slow	slow	1	1
	Microsome	very slow	very slow	1	1
Amitriptyline	Homogenate	very slow	very fast	4 (1)	9 (4)
	Microsome	very slow	very fast	4 (1)	9 (5)
Digoxin	Homogenate	very slow	very slow	none	none
	Microsome	very slow	very slow	none	none
Orphenadrine	Homogenate	slow	fast	3	4 (1)
	Microsome	slow	moderate	3	4 (1)
Propranolol	Homogenate	moderate	fast	5 (1)	7 (3)
	Microsome	slower	slow	5 (1)	7 (3)

Tab. 1: Comprehensive categorical survey of the results (continued)

		Apparent disappearance[1]		Metabolites formed[2]	
Compound	Preparation	Human	Rat	Human	Rat
Methadone	Homogenate	slow	moderate	1 (1)	1 (1)
	Microsome	slow	moderate	1 (1)	1 (1)
Thioridazine	Homogenate	moderate	moderate	11 (1)	17 (3)
	Microsome	moderate	moderate	11 (1)	17 (3)
Maprotiline	Homogenate	slow	moderate	1	4 (1)
	Microsome	slow	slow	4	9 (3)
Diphenhydramine	Homogenate	slow	moderate	3	6 (1)
	Microsome	slow	moderate	3	6 (2)
Haloperidol	Homogenate	slow	slow	6 (2)	6 (2)
	Microsome	slow	slow	6 (2)	7 (3)
Atropine	Homogenate	very slow	fast	3	5 (1)
	Microsome	very slow	slow	2	5 (1)
Disopyramide	Homogenate	slow	slow	1	3
	Microsome	very slow	slow	1	3
Diphenylhydantoin	Homogenate	very slow	slow	1	1
	Microsome	very slow	slow	1	1
Warfarin	Homogenate	slow	slow	3	4
	Microsome	slow	slow	3	3
Chloramphenicol	Homogenate	?	moderate	none	1
	Microsome	?	very slow	none	1
Rotenone	Homogenate	moderate	fast	7	7
	Microsome	moderate	moderate	8	8
Diethylphalate	Homogenate	very fast	very fast	1	1
	Microsome	very fast	very fast	1	1
Dibutylphtalate	Homogenate	very fast	very fast	2 (1)	2 (1)
	Microsome	very fast	very fast	2 (1)	3 (1)
Ibuprofen	Homogenate	fast	moderate	1	1
	Microsome	moderate	moderate	1	1
Gibberellic acid	Homogenate	no met	very slow	none	none
	Microsome	no met	no met	none	none
Propylparaben	Homogenate	very fast	very fast	2 (1)	2 (1)
	Microsome	very fast	very fast	3 (1)	3 (1)
Nicotine	Homogenate	moderate	moderate	3 (1)	1
	Microsome	slow	slow	3 (1)	none
Quinidine	Homogenate	moderate	moderate	5 (1)	6 (1)
	Microsome	slow	slow	5 (1)	5 (1)
Verapamil	Homogenate	fast	fast	8 (2)	9 (2)
	Microsome	moderate	moderate	7 (2)	8 (2)
Diazepam	Homogenate	slow	moderate	3 (2)	7 (2)
	Microsome	slow	moderate	3 (2)	6 (3)
Malathion	Homogenate	rapid	rapid	4	4
	Microsome	fast	fast	4	3
Phenobarbital	Homogenate	none	slow	none	none
	Microsome	none	slow	none	none
Pentobarbital	Homogenate	very slow	slow	none	2
	Microsome	very slow	slow	none	1
Fenpropathrin	Homogenate	slow	moderate	3	5
	Microsome	moderate	moderate	4	5
Chlorpyrifos	Homogenate	moderate	moderate	6 (3)	3
	Microsome	moderate	moderate	2 (1)	2 (1)
Carbaryl	Homogenate	moderate	moderate	3 (1)	2
	Microsome	slow	slow	4 (2)	4

Tab. 1: Comprehensive categorical survey of the results (continued)

		Apparent disappearance[1]		Metabolites formed[2]	
Compound	Preparation	Human	Rat	Human	Rat
Cycloheximide	Homogenate	slow	slow	1	none
	Microsome	very slow	very slow	1	none
Nalidixic acid	Homogenate	slow	slow	2 (1)	1
	Microsome	slow	very slow	2 (1)	1
Antipyrine	Homogenate	slow	slow	none	none
	Microsome	very slow	slow	none	none
Caffeine	Homogenate	very slow	very slow	1	none
	Microsome	very slow	very slow	none	none
Theophylline	Homogenate	very slow	very slow none none		
	Microsome	very slow	very slow	none	none
Procainamide	Homogenate	very slow	moderate	1	3(1)
	Microsome	very slow	slow	none	3 (2)
Isoniazide	Homogenate	none	none	none	none
	Microsome	none	none	none	none
Aminopterin	Homogenate	none	none	none	none
	Microsome	none	none	none	none
Aflatoxin B1	Homogenate	moderate	slow	3 (1)	2 (1)
	Microsome	moderate	slow	2 (1)	2 (1)
Colchicine	Homogenate	slow	slow	2 (1)	3 (1)
	Microsome	slow	slow	2 (1)	3 (1)
Strycknine	Homogenate	moderate	moderate	4	6
	Microsome	slow	slow	4	4
Physostigmine	Homogenate	slow	fast	5 (1)	7 (3)
	Microsome	slow	moderate	3 (1)	5 (2)
Fipronil	Homogenate	moderate	slow	1	1
	Microsome	slow	slow	1	1
Dextropropoxifene	Homogenate	moderate	very fast	4 (1)	11 (1)
	Microsome	moderate	fast	4 (1)	11 (1)
Methylphenidate	Homogenate	slow	slow	2 (1)	4 (1)
	Microsome	slow	slow	1	3 (1)
Aminoglutethimide	Homogenate	slow	moderate	1	none
	Microsome	slow	moderate	none	1
Diuron	Homogenate	moderate	slow	2 (1)	1
	Microsome	slow	slow	3 (1)	1
Metalaxyl	Homogenate	moderate	fast	7 (2)	7 (2)
	Microsome	slow	moderate	3 (2)	4 (1)
Fenvalerate	Homogenate	slow?	slow?	1	2
	Microsome	slow?	slow?	2	4
Abamectin	Homogenate	fast	?	5	3
	Microsome	moderate	moderate	6	3
Amphetamine	Homogenate	very slow	very slow	none	none
	Microsome	very slow	very slow	none	none
Busulfan	Homogenate	very slow	very slow	none	none
	Microsome	very slow	very slow	none	none
Paracetamol	Homogenate	very slow	very slow	1	1
	Microsome	very slow	very slow	none	none
Parathion	Homogenate	moderate	fast	1	1
	Microsome	moderate	slow	1	1

[1] Categories for substrate depletion: very slow (<5%), slow (5-19%), moderate (20-50%), fast (50-80%), very fast (>80%) in the first 15-min incubation. Consistency of substrate loss curve was generally assessed on the basis of the whole substrate depletion curve over 60 minutes.

[2] The first figure is the number of all identified metabolites; the figure in parentheses means the number of major metabolites.

Categories for substrate depletion were the following: very slow (<5%), slow (5-19%), moderate (20-50%), fast (50-80%), very fast (>80%) in the first 15-min incubation. Substrate loss was generally estimated on the basis of the whole substrate depletion curve over 60 minutes. Sometimes time-dependent changes were not consistent and a certain degree of uncertainty remains for some compounds.

Metabolites are classified into major and minor metabolites on the basis of relative peak areas. It is of importance to stress that signal strengths may be different for various types of metabolites and consequently, assignments into major and minor metabolites are only provisional.

3.3 Substrate depletion

Metabolic stability was very variable; for a number of substrates, loss was negligible over the incubation time (e.g. digoxin, gibberellic acid), for some others, no substrate was detectable after 15 minutes (e.g. diethyl and dibutyl phthalate, propylparaben). It was possible to calculate an apparent intrinsic clearance for most of the chemicals, although for those at extreme ends (i.e. very stable or labile chemicals) only "limit" clearances (smaller or larger than a certain value) were possible to estimate. In Table 2 we have collected the chemicals which displayed either very slow (<5% depleted) or very fast (>95%) depletion in incubations. However, a more quantitative approach necessitates also a value for the binding of each substance with microsomes or homogenates, to be used for the calculation of unbound intrinsic clearances. Because of large differences in lipid solubility and ionization and other properties of the chemicals studied, measurement of microsomal or homogenate binding for some substances proved very difficult and further investigations have to be performed. It has to be stressed that some substances may bind also to plastic or glass and give erroneous values for the unbound fraction.

Differences between human and rat

With respect to metabolic stability, most chemicals displayed relatively similar behaviour in human and rat preparations, i.e.

Tab. 2: Very slowly (<5 % in 15 minutes, i.e. difficult to reliably study) or very quickly (>95 % in 15 minutes) depleted chemicals in human and rat liver homogenates and microsomes

Chemicals	Remarks
Very Slowly Depleted Compounds	
Human liver homogenates (h)/microsomes (m)	
Carbamazepine	Also rat m
Amitriptyline	
Digoxin	Also rat
Atropine	
disopyramide m	
Diphenylhydantoin	
Gibberellic acid	
Phenobarbital	
Pentobarbital	
Cycloheximide m	Also rat m
Antipyrine m	
Caffeine	Also rat
Theophylline	Also rat
Aminopterin	Also rat
Amphetamine	Also rat
Busulfan	Also rat
Paracetamol	Also rat
Rat liver homogenates/microsomes (not including above mentioned)	
Chloramphenicol m	
Nalidixic acid m	
Quickly depleted chemicals	
Human liver homogenates/microsomes	
Diethyl phthalate	Also rat
Dibutyl phthalate	Also rat
Propylparaben	Also rat
Rat liver homogenates/microsomes	
Amitriptyline	
Dextropropoxiphene h	

differences were either negligible or from minor to moderate at the most. However, there were several notable exceptions, the most conspicuous ones e.g. amitriptyline, aflatoxin B1, dextropropoxifene (in homogenates), and parathion (in homogenates).

Differences between microsomes and homogenates
For most chemicals, differences between microsomes and homogenates in metabolic stability were relatively modest at least in the initial analysis. Exceptions included propranolol, nicotine, quinidine, verapamil, carbaryl (quite large quantitative differences), chloramphenicol, and parathion (only in rat). However, it has to be stressed that on-going calculations of intrinsic clearances employing scaling factors and unbound substrate concentrations may change the initial results at least to a certain extent.

3.4 Metabolite formation

Most of the chemicals were metabolized to one or more metabolites, which were tentatively identified on the basis of the exact mass (Table 1). Because of curation of this huge data base, identifications will be published later and compared with published findings of *in vitro* and in metabolism. For some drugs, close to 10 or even more metabolites were detectable (e.g. thioridazine, verapamil, amitriptyline).

Some chemicals, such as caffeine and theophylline displayed unexpectedly very little metabolism. A potential reason might have been that a low substrate concentration was used. Caffeine and theophylline are known to be low-affinity substrates for their metabolizing enzymes. Another interesting chemical was paracetamol, for which only one metabolite, a sulphate conjugate, was detectable in homogenates, but (expectedly) not in microsomes. Again, a possible reason was a low substrate concentration used.

We have collected the compounds with no detectable metabolites or a metabolite detectable in 1 or 2 preparations in Table 3. Altogether 11 out of 55 compounds did not show any metabolites. Some of these compounds may be genuinely "non-metabolizable", but some others, e.g. antipyrine, caffeine, theophylline, amphetamine and paracetamol are extensively metabolized by the hepatic enzymes. As said above, it is probable that in these cases the reason for the lack of metabolites is due to the low substrate concentration, resulting in a very low production of a metabolite(s) in relations to the sensitivity of the assay.

Differences between human and rat
We have compiled in Table 4 a summary of differences in the presence/absence of metabolites and major/minor metabolites between human and rat, on one hand, between homogenates and microsomes, on the other.

Regarding the metabolites formed, there were mostly quantitative differences, but several compounds displayed also species-dependent qualitative differences, i.e. a metabolite was detectable in one species, but not in the other. Especially noteworthy was that about 20 compounds, i.e. about 30 % of all the substances, displayed a species difference in the formation of major metabolite(s) and in about half of the compounds at least some minor metabolites were different between humans and rats. However, these qualitative differences may also have been a consequence of the sensitivity of the analytical method, and it is possible that different metabolite profiles may have been detected with a more sensitive method. For example, dextropropoxiphene displayed quite striking both quantitative and qualitative differences between human and rat preparations.

Differences between microsomes and homogenates
For most chemicals, differences between microsomes and homogenates were relatively modest at least in the initial analysis. Exceptions included paracetamol (sulphate conjugation is not a microsomal reaction) and metalaxyl (more metabolites pro-

Tab. 3: Chemicals with no detectable metabolites or a metabolite detectable in only 1 or 2 preparations in human and rat liver homogenates and microsomes

Chemicals	Remarks
No detectable metabolites	
Human liver homogenates and microsomes	
Digoxin	Also rat
Gibberellic acid	Also rat
Phenobarbital	Also rat
Pentobarbital	
Antipyrine	Also rat
Caffeine	1 metabolite only in human homogenate
Theophylline	Also rat
Aminopterin	Also rat
Amphetamine	Also rat
Busulfan	Also rat
Paracetamol	1 metabolite detectable only in human and rat homogenate
Rat liver homogenates and microsomes (not including above mentioned)	
Pentobarbital	
Cycloheximide	

Tab. 4: Similarities and differences in the presence/absence and major/minor metabolite(s) between human and rat liver homogenates and microsomes

	Human homogenate vs microsomes	Rat homogenate vs microsomes	Homogenate human vs rat	Microsomes human vs rat
No metabolites detectable	10	10	8	10
metabolite(s) in one, but not in the other	5	3	6	6
only one metabolite	8	9	7	6
major metabolite(s) same	21	18	14	14
major metabolite(s) different	10	15	20	17
minor metabolite(s) same	11	7	2	2
minor metabolite(s) different	18	22	28	28

duced by homogenate). Again here, the sensitivity of the analytical method is of importance. However, as in the case of differences between human and rat, the mere counting of similar or different metabolites may create a wrong impression about the significance of these differences. It may well be that one or two metabolites which are different between human and rat, or homogenates and microsomes, will not lead to any "real" or "really relevant" biological or toxicological consequences.

4 Conclusions

Several important conclusions can be made from the above described results, even before further research towards in-depth analyses and *in vitro-in vivo* extrapolations.

1) LC-MS –based analytical methods are efficient in the primary *in vitro* phase of studying organic chemicals, for both measuring the disappearance of the parent compound and the formation and tentative identification of metabolites. However, selection of chemicals for LC-MS analysis has to be made based on the type of instrument available.
2) Only 4 out of 55 preselected ECVAM/ICCVAM validation chemicals had to be changed because of analytical problems. All of them were highly halogenated compounds with poor detectability in the MS analysis.
3) Several widely spaced concentrations of the studied substance should be preferably used, even in the face of increasing costs of analyses. In this feasibility study we had to select, for cost reasons, only one concentration, 25 uM, which proved to be too low for many low-affinity compounds such as caffeine, theophylline and carbamazepine. In the *in vitro* phase of studies, it may not be wise to select concentrations which are in the range of anticipated *in vivo* concentrations.
4) A preliminary comparison of microsomes and homogenates indicated that differences were not large for most of the substances. However, it has to be recognized that microsomes as an enzyme source would not produce most phase II metabolites (e.g. paracetamol-sulphate).
5) For most compounds, microsomes are still suitable for stability and metabolism screening, but it is difficult to anticipate the extent and significance of wrong conclusions, consequent to the selection of microsomes over homogenates as an enzyme source.
6) A tentative categorical analysis indicated that differences between human and rat preparations were rather modest for most of the substances. There were a number of exceptions, e.g. amitriptyline and aflatoxin B1 regarding substrate loss. Qualitative differences in metabolite profiles were relatively common, about a third of compounds displayed a difference in major metabolite(s) and in about a half of the compounds some minor metabolites were different. However, most of these differences may not be too important. Differences were usually quantitative.
7) A tentative conclusion is that for most compounds, interspecies extrapolation between rat and human is anticipated to be relatively feasible and reliable. However, before the complete analysis of this huge data base, it is difficult to predict those compounds displaying interspecies differences of such magnitude that important pharmacological or toxicological consequences would ensue.

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