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An extensive cocktail approach for rapid risk assessment of *in vitro* CYP450 direct reversible inhibition by xenobiotic exposure

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ABSTRACT

Acute exposure to environmental factors strongly affects the metabolic activity of cytochrome P450 (P450). As a consequence, the risk of interaction could be increased, modifying the clinical outcomes of a medication. Because toxic agents cannot be administered to humans for ethical reasons, *in vitro* approaches are therefore essential to evaluate their impact on P450 activities.

In this work, an extensive cocktail mixture was developed and validated for *in vitro* P450 inhibition studies using human liver microsomes (HLM). The cocktail comprised eleven P450-specific probe substrates to simultaneously assess the activities of the following isoforms: 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 2J2 and subfamily 3A. The high selectivity and sensitivity of the developed UHPLC-MS/MS method were critical for the success of this methodology, whose main advantages are: (i) the use of eleven probe substrates with minimized interactions, (ii) a low HLM concentration, (iii) fast incubation (5 min) and (iv) the use of metabolic ratios as microsomal P450 activities markers. This cocktail approach was successfully validated by comparing the obtained IC_{50} values for model inhibitors with those generated with the conventional single probe methods. Accordingly, reliable inhibition values could be generated 10-fold faster using a 10-fold smaller amount of HLM compared to individual assays. This approach was applied to assess the P450 inhibition potential of widespread insecticides, namely, chlorpyrifos, fenitrothion, methylparathion and profenofos. In all cases, P450 2B6 was the most affected with IC_{50} values in the nanomolar range. For the first time, mixtures of these four insecticides incubated at low concentrations showed a cumulative inhibitory *in vitro* effect on P450 2B6.

1. Introduction

Cytochromes P450 (P450) are the major phase I metabolic enzymes involved in the oxidative biotransformation of xenobiotics as well endogenous compounds. High inter-individual differences in P450 activities have been described in many publications, and major sources of this variability are associated with genetic features (*e.g.*, gene expression regulation, polymorphism, gender, and age), environmental influences (*e.g.*, stress, diet, and life style) and/or xenobiotic exposure (*e.g.*, drug therapy, dietary supplements, environmental pollutants, and toxic substances). As a consequence, the perturbations of their activities (inhibition/reduction or increase/induction) may lead to a significant variation in the concentration of a xenobiotic and its metabolites at the target site, *i.e.*, enhanced clearance, production of toxic metabolites or toxic accumulation of the parent compound. The inhibition of P450 has been shown to produce unexpected severe effects in drug pharmacokinetics and clinical responses, particularly due to drug-drug interactions (DDIs) (Zanger and Schwab, 2013).

The evaluation of the P450 interaction potential of a xenobiotic must thus be conducted to predict the risks in the case of co-exposure. DDI investigations in the early discovery process have already been achieved to reduce the frequency of costly late failures of the drug candidates and to promote safer medical treatment. In contrast to the clinical environment, where DDIs could also be investigated *in vivo* thanks to dedicated clinical studies, drug-toxicant interactions cannot be easily performed for evident ethical reasons. However, because toxicants are ubiquitous in the environment and humans are chronically and/or acutely exposed throughout their entire lives, their impact on P450 activities should be more thoroughly investigated, even if official recommendations in this context are not yet enforced (Pelkonen et al., 2008).

According to the 3Rs principle (Replace, Reduce and Refine) for promoting a more reasonable and ethical animal testing, *in vitro* approaches have been further recommended to evaluate and/or anticipate the impact of toxicants on P450 activities. Hepatocytes are

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currently the gold standard *in vitro* tool for performing P450 induction studies, whereas for studying the inhibition phenomenon at the enzyme level, human liver microsomes (HLM) can be judiciously employed as an *in vitro* native phase I enzyme source thanks to their commercial availability, ease of handling and reliable *in vivo* extrapolation (Parkinson et al., 2010).

Nowadays, except for numerous academic settings, *in vitro* P450 inhibition assays are generally conducted using higher-end robotic systems or high-throughput-MS methods (*e.g.*, RapidFire® and Phytronix®), which drastically increase the efficiency of the assays (Haarhoff et al., 2016; Wu et al., 2007; Wu et al., 2012). Alternatively, the cocktail approach has been developed to efficiently and rapidly monitor the activities of several microsomal P450 isoforms within a single test, reducing the time and assay costs when expensive robotic handling instruments are not available (Lahoz et al., 2008). However, when an extended substrate mixture is used for an overall P450 inhibition screening, the following numerous challenges have been identified: (i) an enhanced risk of probe-probe interactions, (ii) a more difficult implementation according to the different optimal incubation conditions for both rapid-and slow-turnover substrates and (iii) the need for efficient separation-based analytical techniques such as LC/MS methods for the reliable analysis of the high number of analytes. Above all, the combination of high-turnover substrates together with low-turnover substrates has often been recognized as the major issue for implementing unique optimal incubation conditions. Indeed, to allow the detection of low amounts of a metabolite, a relatively high concentration of a slow-turnover substrate along with a high protein concentration and incubation time have to be employed. This practice indirectly promoted the occurrence of probe-probe interactions and simultaneously created disadvantaged conditions for high-turnover substrates. Due to the presence of probe interactions not fully characterized as well as sub-optimal assay conditions for each substrate, the reliability of inhibition investigations could be reduced (Spaggiari et al., 2014a). Recently, the separation of critical substrates into two distinct cocktails has been successfully proposed to overcome probe interactions and facilitate cocktail design. Authors also highlighted the importance of chromatographically separating metabolites and substrates still present in the incubation mixture prior to analysis to avoid potential and relevant analytical interferences due to co-elution, which could not be adjusted using a single analytical standard. Besides pooled samples could be analyzed to save analysis time, this methodology duplicated reagent consumption and manipulations (Dinger et al., 2014a). Alternatively, with the powerful separation capabilities of ultra-high-pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), including its high sensitivity, selectivity and resolution, it was possible to overcome some of the difficulties of cocktail approach. Analytical interferences were corrected using stable isotope-labeled metabolites to correct potential co-elutions (Kozakai et al., 2012).

In this study, an alternative analytical strategy was developed for the original design of an extensive cocktail assay for a microsomal P450 direct reversible inhibition screening (IC_{50} assay) for the most important isoforms involved in the biotransformations of clinically used drugs, namely, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 2J2 and subfamily 3A. An optimized UHPLC separation of the eleven substrates and their P450-specific metabolites was combined with highly sensitive MS/MS detection to achieve favorable analytical and metabolic conditions for optimal cocktail incubation. As a novel aspect, P450 microsomal activities were expressed by combining metabolite formation with substrate depletion (*i.e.*, metabolic ratio) rather than metabolite formation only in the analysis. Afterwards, the inhibition curves and subsequent IC_{50} values were compared to those obtained with the single probe approach. Finally, this assay demonstrated to be a promising safety assessment tool for the evaluation of environment-related direct reversible inhibition of P450 (*e.g.*, pesticides, cosmetics, food additives, phytochemicals, diet, *etc.*), which is currently not systematically addressed in the panel of toxicological screening tests.

2. Materials and methods

2.1. Chemicals, reagents, test compounds and other materials

Acetonitrile (MeCN), methanol (MeOH) and water of ULC/MS grade were purchased from Biosolve (Valkenswaard, Netherlands). Formic acid was obtained from Merck (Darmstadt, Germany). (*S*)-(+)-*N*-3-benzylnirvanol (98%), chlorzoxazone (98%), *O*-desmethylastemizole (98%) hydroxybupropion (95%), 6-hydroxychlorzoxazone (97%), 5-hydroxyomeprazole (98%) and omeprazole (98%) were purchased from Toronto Research Chemicals (Ontario, Canada). Acetaminophen (99%), ammonium hydroxide, amodiaquine dihydrochloride dihydrate (98%), astemizole (98%), bupropion hydrochloride (98%), coumarin (99%), dextromethorphan hydrobromide (99%), dextrorphan tartrate (98%), diclofenac sodium salt (98.5%), flunarizine dihydrochloride (98%), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (99.5%), 7-hydroxycoumarin (99%), 4′-hydroxydiclofenac (98%), ketoconazole (98%), phenacetin (97%), potassium hydroxide, quercetin dihydrate (98%) quinidine anhydrous (98%), testosterone (99%), thio-TEPA (98%) and *N*-desethylamodiaquine dihydrochloride stock solution (1.0 mg/ml in MeOH of free base) were obtained from Sigma-Aldrich (Buchs, Switzerland). Dimethyl sulfoxide (Me_2SO), α-naphthoflavone (99.5%) and 4-methylpyrazole (97%) were bought from Acros Organics (Wohlen, Switzerland) and methoxsalen (99%) was obtained from Alfa Aesar (Schiltigheim, France). Stock solutions of midazolam and 1-hydroxymidazolam and 6β-hydroxytestosterone (1.0 mg/ml in MeOH of free base) were purchased from Lipomed (Arlesheim, Switzerland), whereas stock solutions of chlorpyrifos, fenitrothion, methylparathion and profenofos (1.0 mg/ml in acetone of free base) were kindly given by the Official Food and Veterinary Control Authority of the state of Geneva (Switzerland). β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH) (96%) was obtained from Applichem (Darmstadt, Germany). Sulfaphenazole (99%) and pooled human liver microsomes (pHLM) from 30 donors were purchased from Corning Life Sciences (Amsterdam, the Netherlands). Aliquots of pHLM were stored at −80 °C. A 50 mM NADPH stock solution was prepared in water and stored at −20 °C. Immediately before the microsomal assay, intermediate concentrations of pHLM were prepared by diluting aliquots with the 50 mM HEPES buffer solution, which was prepared by dissolving the required amount of HEPES in water. The pH of HEPES buffer was adjusted to 7.4 with potassium hydroxide using a Seven Multi S40 Mettler Toledo pH meter (Greifensee, Switzerland).

2.2. Characterization of microsomal P450 activities

2.2.1. Preparation of stock solutions and working solutions for inhibition experiments. Stock solutions of substrates and metabolites were prepared in MeOH at 1 mg/ml (free base) and stored at −20 °C for 6 months or less according to their solubilities and stabilities. Intermediate solutions were appropriately reconstituted daily in the 50 mM HEPES buffer solution (pH 7.4) (Spaggiari et al., 2014b). The concentrations of stock solutions of model inhibitors were individually optimized according to their tested concentration range, dilution factor and solubility. In any case, the percentage of the organic solvent in the final incubation mixture did not exceed 1% (*v*/*v*) (Spaggiari et al., 2014a). Such solutions were prepared as follows: α-naphthoflavone (Me_2SO, 4 mM), methoxsalen (MeOH, 4.6 mM), thio-TEPA (MeOH, 5.3 mM), quercetin (Me_2SO, 20 mM), sulfaphenazole (MeOH, 3.2 mM), (*S*)-benzylnirvanol (MeOH, 3.4 mM), quinidine (MeOH, 3.1 mM), 4-methylpyrazole (MeOH, 12.2 mM), flunarizine (MeCN/MeOH 1:1 *v*/*v*, 100.5 mM), and ketoconazole (MeOH, 1.9 mM). Because acetone is not an appropriate organic solvent for *in vitro* P450-mediated metabolism investigations, stock solutions of insecticides were reconstituted in Me_2SO at 30 mM (Spaggiari et al., 2014a). The model inhibitor and insecticide stock solutions were serially diluted daily in the 50 mM HEPES

buffer solution (pH 7.4) to the expected intermediate concentrations before incubation.

2.2.2. Microsomal incubation protocol. Incubations with pHLM were performed in duplicate with a final incubation volume of 100 µl (Spaggiari et al., 2014b, 2014c). The reaction medium contained 50 mM HEPES (pH 7.4), an excess of NADPH, 0.3 mg protein/ml of pHLM, an individual P450 probe substrate or a cocktail of substrates and the inhibitor. The final substrate concentrations were reached by adding the required volume of the intermediate substrate or cocktail solution to the reaction medium. These concentrations were as follows: 1 µM for phenacetin, 2 µM for coumarin, 0.5 µM for bupropion, 0.025 µM for amodiaquine, 1 µM for diclofenac, 1 µM for omeprazole, 1 µM for dextromethorphan, 5 µM for chlorzoxazone, 1 µM for midazolam and 5 µM for testosterone. The final organic solvent concentration was kept lower than 1% (*v*/*v*). After pre-incubation for 3 min at 37 °C, the P450-dependent phase I reactions were initiated by adding an excess of NADPH (2.0 mM). The incubation proceeded during 5 min at 37 °C under agitation (400 rpm). The enzymatic reaction was stopped by adding 100 µl of ice-cold MeCN to the reaction medium. The metabolic ratio (*i.e.*, metabolite to substrate peak area ratio) was employed for analytical variability correction instead of the use of an analytical standard (Spaggiari et al., 2014b). The precipitated proteins were removed by centrifugation (10 min at 14,000 rpm, 10 °C), and an aliquot (150 µl) of the resulting supernatant was transferred to a vial for LC/MS analysis.

2.2.3. Incubation with model inhibitors. Model P450 inhibitors were incubated in duplicate as previously described. For assessing model inhibitor and probe reactions P450 specificities in the current metabolic system, targeted inhibitor concentrations were introduced with the cocktail of substrates as follows: naphthoflavone 1 µM for P450 1A2, methoxsalen 1 µM for P450 2A6, thio-TEPA 30 µM for P450 2B6, quercetin 3 µM for P450 2C8, sulfaphenazole 10 µM for P450 2C9, (*S*)-benzylnirvanol 1 µM for P450 2C19, quinidine 1 µM for P450 2D6, 4-methylpyrazole 30 µM for P450 2E1, flunarizine 30 µM for P450 2J2 and ketoconazole 1 µM for the P450 3A subfamily (Khojasteh et al., 2011; Zhang et al., 2007).

For the determination of inhibition curves using the single substrate and the cocktail, seven inhibitor levels were chosen in the subsequent concentration ranges: α-naphthoflavone [0.01–10 µM] for P450 1A2, methoxsalen [0.01–10 µM] for P450 2A6, thio-TEPA [0.3–300 µM] for P450 2B6, quercetin [0.1–100 µM] for P450 2C8, sulfaphenazole [0.01–10 µM] for P450 2C9, (*S*)-benzylnirvanol [0.03–30 µM] for P450 2C19, quinidine [0.01–10 µM] for P450 2D6, 4-methylpyrazole [0.03–30 µM] for P450 2E1, flunarizine [1–1000 µM] for P450 2J2 and ketoconazole [0.003–3 µM] for the P450 3A subfamily.

2.2.4. Incubation with toxicants. Single-point inhibition was assessed by incubating insecticides at 10 µM in duplicate using the same protocol as described in Section 2.2.2. The inhibition curves were obtained by incubating the cocktail of substrates with eleven different inhibitor concentrations ranging from 0.003 µM to 300 µM.

2.2.5. Data treatment. Prism v. 5.01 (GraphPad Software, San Diego, CA, USA) software was employed to determine the most appropriate fitting model for the depletion of the eleven substrates (one phase decay), the formation of the eleven metabolites (one site-total) and the trend of the metabolic ratio over time (linear or quadratic) during cocktail design.

Activity levels were expressed as percentages (%) and assessed by dividing the metabolic ratio (metabolite to substrate peak area ratio) obtained in the presence of inhibitor with the average of duplicate values of the metabolic ratios obtained in the control samples (solvent only). The results from duplicate incubations are presented as the average ± range. The inhibition curves were obtained by plotting the remaining P450 activity (%) over the logarithm of the inhibitor concentrations. Best-fit IC_{50} values obtained independently with the cocktail approach or single probe approach were compared using an *F-test* (extra sum-of-squares) and were considered to be significant with a p value lower than 0.05.

For radar chart representation, the average of duplicate values of the metabolic ratios obtained in the presence of inhibitor is expressed as the percentage of the average of the metabolic ratios obtained in duplicate for control samples on a logarithmic scale, as previously described (Spaggiari et al., 2014b).

2.3. Liquid chromatography-mass spectrometry

2.3.1. UHPLC-QqQ/MS instrumentation. The experiments were carried out using an Agilent Infinity 1290 ultra-high-pressure liquid chromatography (UHPLC) system (Agilent Technologies, Waldbronn, Germany). The instrument was equipped with a binary pump with a maximum delivery flow rate of 5 ml/min (up to 1200 bar), an autosampler including a flow-through needle, a flexible cube enabling the rinsing of the needle seat and a column compartment thermostated at 33 °C. The UHPLC system was coupled in an optimized configuration with an Agilent 6490 triple quadrupole mass spectrometer (QqQ/MS) equipped with an Agilent Jet Stream electrospray ionization source (Rodriguez-Aller et al., 2013). The samples were stored at 4 °C in the autosampler prior to and during the analysis. Data acquisition, treatment and instrument control were performed using Mass Hunter version B 06–00 SP2 (Agilent Technologies, Waldbronn, Germany).

2.3.2. RPLC conditions. Optimal separation was performed with a Waters (Milford, Massachusetts, USA) Acquity UPLC BEH Shield RP18 column (1.7 µm, 2.1 mm × 50 mm) and a flow rate of 500 µl/min. Gradient elution (solvent A, 10 mM ammonium formate buffer (pH 3.7); solvent B, MeOH) was used according to the following method: 2–95% solvent B in 5 min, held at 95% solvent B to 6.0 min, and column reconditioning at 2% solvent B to 7 min (total analysis time). The injection volume was 2 µl. For modeling retention times, DryLab®2010 Plus modeling software (Molnar-Institute, Berlin, Germany) was employed.

2.3.3. ESI-QqQ/MS parameters. ESI was operated alternatively in both the positive and negative modes with polarity switching (20 ms delay). The optimal selected reaction monitoring (SRM) traces (precursor ion, production ion and collision energy) for each compound are reported in Table 1, along with other compound-related (collision cell accelerator voltage, dwell time), source, ion funnel and multiplier detector parameters. The optimal fragmentor voltage was set to 380 V during autotuning of the instrument. The interchannel delay between the two SRM traces was 2 ms (Rodriguez-Aller et al., 2013). The time *filter width* parameter was adjusted to a peak width of 0.02 min, which is the lowest value supported by the software (Spaggiari et al., 2013).

2.3.4. Quantitative evaluation. The response functions for the eleven substrates and their main metabolites ($N = 22$) were evaluated using microsomal incubation mixtures (without cofactor) reconstituted at concentrations ranging from 0.001 ng/ml to 800 ng/ml. To evaluate the linearity of the response function of each specific SRM transition, twenty concentration levels ($k = 20$) injected in triplicate were investigated for each analyte, and the lower limit of quantification (LLOQ) as well as the upper limit of quantification (ULOQ) were defined. The LLOQ was measured as the concentration possessing a response with a signal-to-noise (S/N) ratio superior or equal to 10. The ULOQ was defined as the highest value of the concentration range where the least-squared linear (without weighting) and quadratic regressions are not statistically different. Therefore, the response function linearity ranged from LLOQ to ULOQ. Conventional least-squared linear model

Table 1
Substrates, metabolites, and ESI source parameters for MS/MS detection and cocktail assay.

Substrates and metabolites parameters[a]

P450 isoform	Probe substrate P450-specific metabolite	ESI +/−	Precursor ion *m/z Q1 unit resolution*	Product ion *m/z Q2 unit resolution*	CE [eV]	CAV [eV]	Dwell time [ms]	Time segment	P450-specific reaction and K_m[b] [μM]	Concentration for incubation [nM]	Linear range (LLOQ to ULOQ, $R^2 > 0.99$) [nM]
1A2	Phenacetin	+	180	110	10	2	1	3	*O*-Deethylation	1000	2–2800
	Acetaminophen	+	152	110	10	4	100	1	1.7–152		3–3300
2A6	Coumarin	+	147	91	5	7	50	3	7-Hydroxylation	2000	68–2800
	7-Hydroxycoumarin	+	163	107	10	7	20	3	0.3–2.3		6–2500
2B6	Bupropion	+	240	184	5	4	1	3	Hydroxylation	500	1–2300
	Hydroxybupropion	+	256	238	5	4	10	3	67–168		0.8–400
2C8	Amodiaquine	+	356	283	25	1	100	2	*N*-Deethylation	25	0.14–600
	N-Desethylamodiaquine	+	328	283	5	1	125	2	2.4		0.06–600
2C9	Diclofenac	+	296	214	34	3	40	5	4′-Hydroxylation	1000	0.3–1300
	4′-Hydroxydiclofenac	+	312	230	36	2	40	5	3.4–52		0.2–1300
2C19	Omeprazole	+	346	168	5	2	1	4	5-Hydroxylation	1000	3–1700
	5-Hydroxyomeprazole	+	362	214	5	4	20	3	17–26		0.2–1600
2D6	Dextromethorphan	+	272	215	20	4	1	3	*O*-Demethylation	1000	0.8–1100
	Dextrorphan	+	258	157	44	1	10	3	0.4–8.5		0.07–250
2E1	Chlorzoxazone	−	168	132	19	1	70	4	6-Hydroxylation	5000	3–3600
	6-Hydroxychlorzoxazone	−	184	120	18	2	50	3	39–157		2–3200
2J2	Astemizole	+	459	135	42	2	40	4	*O*-Demethylation	300	0.03–450
	O-Desmethylastemizole	+	445	204	32	5	20	3	0.94[c]		0.05–450
3A subfamily	Midazolam	+	326	291	10	7	5	4	1′-Hydroxylation	1000	3–1300
	1′-Hydroxymidazolam	+	342	324	16	5	40	5	1–14		0.9–1700
	Testosterone	+	289	123	25	4	1	5	6β-Hydroxylation	5000	17–2800
	6β-Hydroxytestosterone	+	305	269	15	1	70	4	52–94		3–2000

ESI source and common detection parameters[d]

Time segment	Time [min]	ESI +/−	Delta EMV [V]	Capillary [V]	Nozzle [V]	HRF[e] [V]	LRF[e] [V]	Nebulizer gas [°C]	Nebulizer gas [L/min]	Nebulizer [psi]	Sheat gas [°C]	Sheat gas [L/min]	cycle/s
1	0–1.4	+	400	2500	0	120	100	250	14	50	400	12	9.66
2	1.4–1.9	+	400	3000	0	120	80	250	14	0	400	12	4.31
3	1.9–2.9	+	400	3000	0	120	100	250	14	50	400	12	3.36
		−	400	4000	0	120	80						
4	2.9–3.4	+	400	4000	0	120	80	250	14	50	400	12	3.53
		−	400	4000	0	160	80						
5	3.4–7.0	+	400	2500	0	90	80	250	14	50	400	12	7.41

[a] CAV, cell acceleration voltage; CID, collision energy; ESI, electrospray ionization; Q, quadrupole; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification.
[b] http://www.fda.gov/drugs/developmentapprovalprocess/developmentresources/druginteractionslabeling/ucm093664.htm (accessed 01/2016).
[c] From Matsumoto et al. (2002).
[d] EMV, multiplier voltage; HRF, high pressure funnel; LRF, low pressure funnel.
[e] iFunnel parameters.

evaluation was achieved for each compound within the expected analyte range, starting from lower LLOQ to ULOQ. For all of the compounds, the adjustment was found to be adequate considering a determination coefficient (R^2) superior to 0.99 over at least three orders of magnitude. All of the LLOQ values were set to allow the required sensitivity for detecting the lowest amount of metabolite produced at a high concentration of inhibitor (*e.g.*, *N*-desethylamodiaquine). Conversely, higher substrate concentrations detected at a high concentration of inhibitor were evaluated below the ULOQ.

For both cocktail and single probe approaches, the intra-assay precision was measured by triplicate injections of each sample ($k = 48$) used for the generation of inhibition curves (control and seven different levels of model inhibitor concentrations, $n = 2$ each). The variability of the eleven metabolic ratios was then evaluated at each level. The quadratic means (or root mean squared values, RMSs) of the relative standard deviations (RSDs) were employed for assessing analytical and incubation method variabilities at specific and global levels (Spaggiari et al., 2014b). To assess the stability of the overall bioanalytical process, inter-assay variability of the eleven metabolic ratios was measured including five cocktail control samples (solvent only) used for IC_{50} assessment and prepared using distinct aliquots of pHLM, fresh daily working solutions and analyzed on different days.

3. Results and discussion

3.1. Cocktail composition and analytical method development

For the first time in the context of the cocktail approach, eleven P450 probe substrates, namely, phenacetin (1A2), coumarin (2A6), bupropion (2B6), amodiaquine (2C8), diclofenac (2C9), omeprazole (2C19), dextromethorphan (2D6), chlorzoxazone (2E1), astemizole (2J2), midazolam and testosterone (3A subfamily), have been selected for simultaneously monitoring the most clinically and toxicologically relevant P450 isoforms in HLM. According to our recent survey of the published *in vitro* cocktails, these substrates were selected in relation to the following advantages: (i) acceptance by regulatory authorities, (ii) a highly specific P450-reaction for the concerned metabolite, (iii) a major metabolic pathway with sufficient substrate turnover in HLM, (iv) good analytical monitoring for both the substrate and metabolite, and (v) reduced probe interaction potential through concentration adjustments (Spaggiari et al., 2014a).

For a relative characterization of activities of P450 and UGT in pHLM, the metabolic ratio, which presents the CYP-specific metabolite concentration at the rate of the probe substrate concentration, has been recently demonstrated as a reliable parameter (Gradinaru et al., 2015; Spaggiari et al., 2014a). Moreover, the latter correct analytical variability

and is particularly adapted for comparative assays, such as inhibition screening studies. However, when dealing with many compounds as in the cocktail approach, the risk for analytical interferences due to possible substrate-substrate, substrate-metabolite or metabolite-metabolite co-elutions increases drastically. For this reason, particular attention must be given to obtain the best chromatographic separation to minimize potential ionization suppression or enhancement at the MS source due to analyte co-elution (Remane et al., 2010). By considering the high number of analytes to separate, a retention time modeling approach has been considered to get the best reversed-phase based UHPLC-MS/MS method able to analyze the eleven substrates and their respective specific metabolites. A generic screening of UHPLC conditions was initially performed to select the best combination of stationary phase chemistry, organic solvent, buffer pH and temperature (Spaggiari et al., 2014c). Regarding the selection of the stationary phase, the Acquity Shield RP18 provided better peak shapes for the strongest basic compounds amodiaquine, astemizole and their P450-specific metabolites due to the electrostatic repulsion provided by the embedded carbamate group in the bonded phase ligand. Chromatographic optimization was then calculated, and the optimal separation was obtained with MeOH and ammonium formate buffer (pH 3.7). The proposed analytical conditions were found to generate the best separation in terms of the retention of the compounds, chromatographic selectivity, peak shapes and sensitivity (S/N), unfortunately without baseline separation of the twenty-two analytes. The best separation in the shortest time was obtained using a gradient from 2% to 95% MeOH in 5 min. As depicted in Fig. 1, only two critical chromatographic pairs were still observed as being co-eluted, namely, *O*-demethylastemizole/dextromethorphan and midazolam/chlorzoxazone, but analytical interferences in MS detection were excluded, as discussed later in the manuscript.

Due to the risk of metabolic interactions between substrates of this extended cocktail, the achievement of a high detection sensitivity was mandatory, particularly for metabolites and substrates incubated at very low concentrations (Spaggiari et al., 2014d). Except for omeprazole and testosterone, the most sensitive SRM transitions were systematically identified, and source and related MS parameters were optimized for each analyte. For compounds whose analytical sensitivity represented a crucial issue for inhibition studies, dwell times (DTs) were increased to the maximal values (*e.g.*, 125 ms) thanks to an MS acquisition divided into five distinct time windows. The cycle time of each time window (which included DTs, interchannel and polarity switching delays) was optimized to be compatible with UHPLC narrow peaks (averaged peak width of approximately 4 s at 4.4% of the peak height) to ensure sufficient data acquisition points for reliable relative quantification. The optimized parameters for the detection of all compounds along with the source parameters, concentrations for incubation and the linear ranges are reported in Table 1.

3.2. Optimization of the incubation conditions for the cocktail assay

According to the high sensitivity achieved with the developed UHPLC-MS/MS method, preliminary studies showed that substrate incubation was possible using a concentration lower than 0.5 mg/ml protein, *i.e.*, the most employed value for cocktail assays using HLM (Spaggiari et al., 2014a). At the concentration of 0.3 mg/ml, the detection of all of the metabolites was still sufficient to allow IC_{50}-based inhibition studies. The linearity of metabolite formation was fully respected, and non-specific binding to the incubation matrix was potentially reduced. Furthermore, because the conventional incubation time of 20 min could be set to 5 min, it represents to our knowledge the fastest incubation time for an extended cocktail approach (Kozakai et al., 2012; Spaggiari et al., 2014a). As depicted in Fig. 2, the following advantages were realized: a reduced substrate depletion (<30%) for avoiding *inter alia* potential product inhibition, except for the substrate coumarin, which presented a very rapid turnover, and the linearity over time for both metabolite formation and the metabolic ratio for all the P450 isoforms. Moreover, a reduced depletion of inhibitors, a reduced risk of metabolite-related interactions and better chemical stability in the incubation mixture were obtained as additional advantages of such rapid incubations using low amounts of enzymes. As recommended, the concentrations of the eleven substrates were initially adjusted near or below their respective K_m values (i) to obtain first-order enzymatic reactions for simply extrapolating the apparent K_i from IC_{50} values, (ii) to selectively activate the high-affinity probe P450-pathway and (iii) avoid potential interactions (Haupt et al., 2015; Spaggiari et al., 2014a). Thanks to the high detection sensitivity, a strong reduction in the substrate concentrations was considered in order to have substrate and product concentrations well below the K_m and K_i values for any reaction. Moreover, this circumstance minimized the percentage of organic solvent needed for better substrate solubility in working solutions. After concentration optimization, the metabolic ratios obtained with single substrate incubations were found to be similar to those obtained with the cocktail approach. All ratios were determined between 90% and 110% of the reference values, which confirmed the lack of both analytical and metabolic interferences in the cocktail mixture. P450 reaction K_m values and final substrate concentrations used for the incubation are reported in Table 1. The use of 25 nM of amodiaquine was mandatory to completely minimize its interaction with several microsomal P450 isoforms. However, this concentration

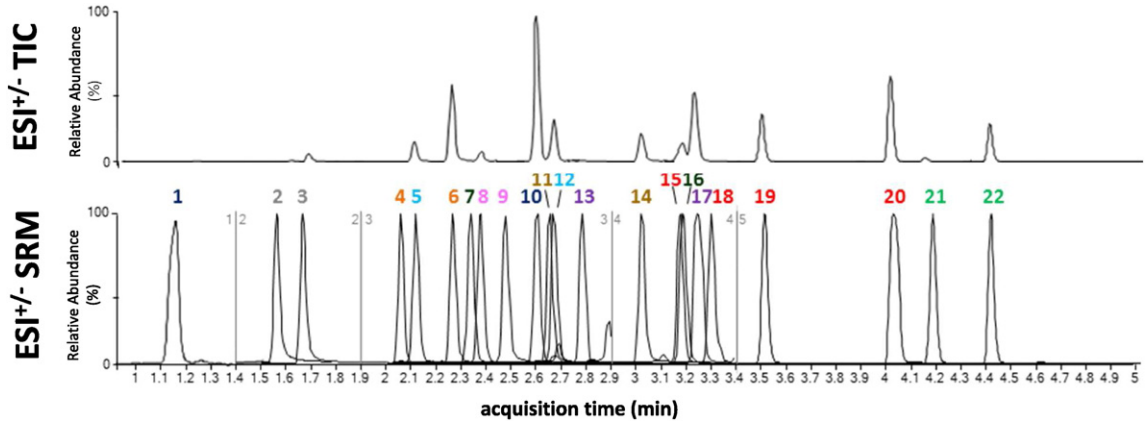

Fig. 1. TIC chromatogram indicating the combination of individual SRM traces and reconstructed extracted ion chromatograms overlaying all the SRM traces. (1) acetaminophen, (2) *N*-deethylamodiaquine, (3) amodiaquine, (4) hydroxybupropion, (5) dextrorphan, (6) bupropion, (7) 6-hydroxychlorzoxazone, (8) 7-hydroxycoumarin, (9) coumarin, (10) phenacetin, (11) *O*-desmethylastemizole, (12) dextromethorphan, (13) 5-hydroxyomeprazole, (14) astemizole, (15) 6β-hydroxytestosterone, (16) chlorzoxazone, (17) omeprazole, (18) midazolam, (19) 1′-hydroxymidazolem, (20) testosterone, (21) 4′-hydroxydiclofenac, (22) diclofenac.

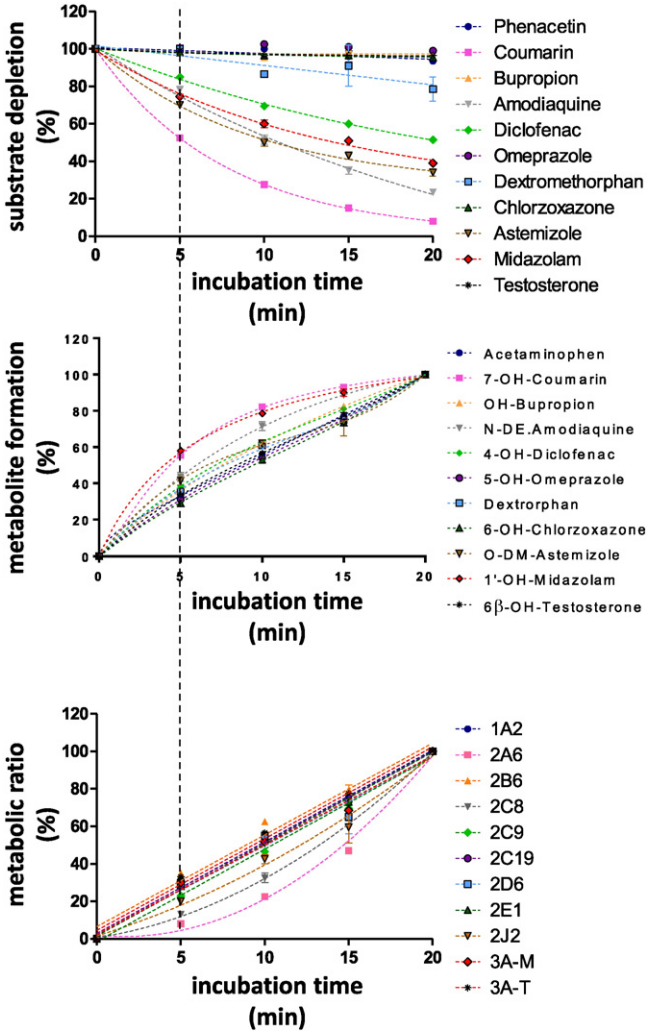

Fig. 2. Probe substrates depletion (%), P450-specific metabolites formation and metabolic ratio trends in pHLM at 0.3 mg protein/ml in function of time.

violated an assumption of Michaelis-Menten steady state kinetics, where the substrate concentration is supposed to be much higher than the catalytic concentration of the enzyme (approximately 15–30 nM). Therefore, if relevant inhibition of P450 2C8 is observed during the screening experiments using the cocktail approach, individual assay incubating amodiaquine at more conventional concentrations (near or equal to K_m values) should be performed to verify the reliability of the observed P450 2C8 inhibition.

3.3. Validation of the cocktail approach

To demonstrate the reliability and the equivalence for measuring IC_{50} values of the proposed cocktail assay with the conventional approach (single substrate incubation), ten model inhibitors were selected, namely, α-naphthoflavone (1A2), methoxsalen (2A6), thioTEPA (2B6), quercetin (2C8), sulfaphenazole (2C9), (*S*)-benzylnirvanol (2C19), quinidine (2D6), 4-methylpyrazole (2E1), flunarizine (2J2) and ketoconazole (3A subfamily). First, the specificities of both the model inhibitors used at recommended *in vitro* phenotyping concentrations and P450-probe reactions have been investigated in the current incubation conditions (Zhang et al., 2007). The most selective model inhibitors were α-naphthoflavone, thioTEPA, sulfaphenazole, (*S*)-benzylnirvanol, quinidine and 4-methylpyrazole. Consequently, P450 1A2, 2B6, 2C19, 2D6 and 2E1 did not contribute to other P450-probe biotransformations and were thus considered to be highly specific in the current incubation conditions. The strong inhibition of both P450 1A2 and 2A6 by methoxsalen was mainly due to the weak selectivity of the model inhibitor more than the contribution of P450 2A6 to phenacetin *O*-deethylation, which was also considered to be highly specific (Khojasteh et al., 2011). A lower selectivity was also observed for quercetin, which strongly inhibited P450 1A2 in addition to 2C8 (Dierks et al., 2001; Khojasteh et al., 2011). Ketoconazole was incubated at the recommended concentration and was found to inhibit several P450 activities, particularly the 2E1-mediated 6-hydroxylation of chlorzoxazone and the 2J2-mediated *O*-demethylation of astemizole, as observed elsewhere (Khojasteh et al., 2011). Although several P450 isoforms could potentially participate in the 6-hydroxylation of chlorzoxazone, its strong inhibition by 4-methylpyrazole confirmed that P450 2E1 is the major isoform involved in these assay conditions. Astemizole *O*-demethylation was recognized to be a major pathway in HLM and a probe reaction for P450 2J2 (Lee et al., 2012; Matsumoto and Yamazoe, 2001). However, P450 2J2 is less expressed in HLM than in intestinal microsomes, which would promote some contribution by P450 2D6 and the 3A subfamily (Matsumoto et al., 2002, 2003). Moreover, it is well known that substrates of P450 2J2 are generally also substrates of the 3A subfamily (Lee et al., 2010). The P450 2D6 should not contribute to astemizole *O*-demethylation because no inhibition by quinidine was observed. On the other hand, ketoconazole strongly affected this reaction, which was consistent with the P450 2J2 inhibition potential of this compound (Lee et al., 2012). Recently, flunarizine was suggested as strong inhibitor of P450 2J2 and 2D6 but not of the 3A subfamily (Ren et al., 2013). In the present study, flunarizine incubated at 30 μM produced only strong inhibition of the microsomal *O*-demethylation of astemizole and the *O*-demethylation of dextromethorphan, which was in agreement with the literature. By combining all of these observations, a major contribution by microsomal hepatic P450 2J2 in these incubation conditions could be accepted. Complete histograms of the phenotyping assay results with error bars are reported in the supporting information (Fig. S1).

Inhibition curves were generated to compare the performance of the cocktail assay with the conventional approach (Fig. 3). The experimental IC_{50} values, interval confidences (95%), *p* values and IC_{50} values from the literature are presented in Table 2 and confirmed the reliability of the proposed cocktail assay for P450 inhibition studies. Only one statistically significant difference ($^*p < 0.05$) between the cocktail and individual IC_{50} values was found with coumarin, the most sensitive substrate to interactions. Compared to other substrates, coumarin was rapidly metabolized into 7-hydroxycoumarin, which produced the highest metabolic ratio (>10) of the cocktail assay. Therefore, weak metabolic and/or analytical interferences could exhibit a higher impact on its metabolic ratio. It has to be noted that both IC_{50} values remain in agreement with data from the literature.

Afterwards, the cocktail variability was assessed as the most important parameter to consider when the relative quantification of inhibition studies is performed (Spaggiari et al., 2014b). The metabolic and analytical variability of the metabolic ratio at each level of the inhibition curve (intra-assay) is shown in the supporting information (Table S1). Considering all of the metabolic ratios, the global analytical variability ranged from 2% to 13%, whereas the overall metabolic variability was between 3% and 17%. The inter-assay variability was evaluated thanks to fresh solutions and different aliquots of HLM, and it included five control incubations (without inhibitor). The P450 2B6 metabolic ratio was found to be the most stable value (9%), whereas the P450 2E1 metabolic ratio exhibited the highest variability (33%). The average variability of the assay was approximately 22% and was considered acceptable for further experiments regarding the screening of toxicants.

3.4. Single-point inhibition by selected toxicants

Four widespread organophosphorothionate (OPs) pesticides, namely, chlorpyrifos, fenitrothion, methylparathion and profenofos,

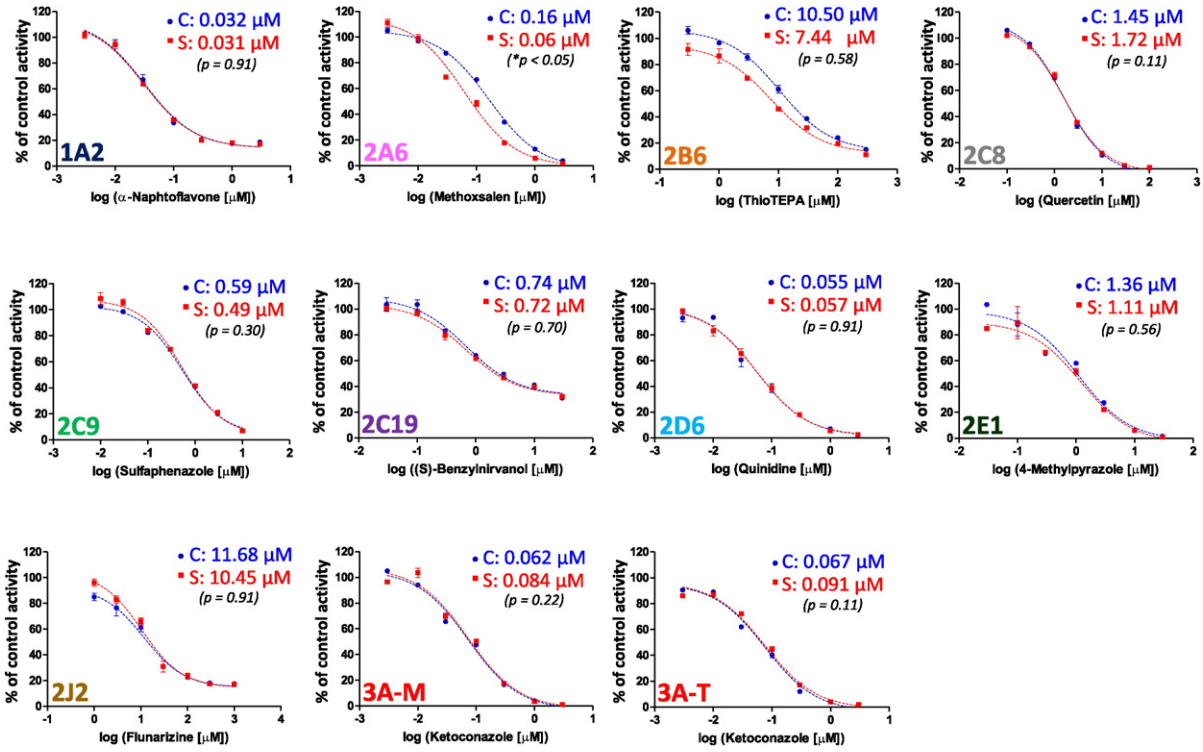

Fig. 3. Inhibition curves obtained using cocktail (C) and single (S) incubation approaches.

were selected to identify their microsomal P450 inhibition potential. A general overview of the most important P450 probe-toxicant interactions was obtained by incubating these selected insecticides at concentrations of 10 µM. This concentration was selected considering specific guidelines for recognizing potent *in vitro* P450 inhibitors (Dierks et al., 2001). According to the observed level of inhibition, results could be extrapolated to human, considered clinically relevant and susceptible to be effective at actual human exposure levels (Buratti et al., 2003). Due to the numerous P450 isoforms monitored, screening results are represented as logarithmic radar charts, already described as an adapted visual tool to rapidly highlight significant effects on P450 activities (Spaggiari et al., 2014b). As illustrated in Fig. 4, the four tested OPs were found to strongly inhibit P450 2B6. In agreement with previous studies, other P450 isoforms were also relevantly affected by the OPs (Abass and Pelkonen, 2012; Di Consiglio et al., 2005). Profenofos exhibited the strongest inhibition of P450 1A2. Another P450 sensitive to OPs was P450 2A6, which was inhibited by 50% when incubated with methylparathion. At this concentration of pesticides, P450 2C19 was also weakly inhibited (40–50%), except by chlorpyrifos.

Because stable isotope-labeled internal standards were not used in this approach for control response, co-eluting test compounds (not monitored by the analytical method) could potentially generate analytical interferences such as ion suppression or enhancement effects that could lead to false positive or false negative inhibition results, respectively. For this reason, incubated control samples terminated with acetonitrile containing test compound were mandatory to check such potential analytical interferences (Dinger et al., 2014a). Irrelevant analytical interferences were observed between the four tested insecticides (10 µM) and the twenty-two monitored analytes.

3.5. Inhibition curves for selected toxicants

Reliable inhibition curves for the ten P450 isoforms were simultaneously assessed by incubating each OP with the cocktail substrates. According to the screening results, an extended range of concentrations was used for each insecticide to have more accurate IC_{50} values, especially for the most affected P450 isoforms, *i.e.*, 2B6, 1A2 and 2C19. As depicted in Fig. 5, for these three P450 isoforms, inhibition curves confirmed their roles in probe-OP interactions. The lowest IC_{50} values were obtained for P450 2B6 and are as follows: 0.13 µM for fenitrothion, 0.18 µM for methylparathion, 0.55 µM for chlorpyrifos and 4.8 µM for profenofos. The P450 1A2 was the second most affected isoform, particularly by profenofos (IC_{50} value of 3.6 µM). The other OPs fenitrothion, chlorpyrifos and methylparathion exhibited IC_{50} values of 5.4 µM, 10.1 µM and 13.7 µM, respectively. P450 2C19 was shown as the minor isoform involved in the interaction with OPs. These values and observations were in agreement with published studies (Abass et al., 2007; Abass et al., 2009; Abass and Pelkonen, 2012; Buratti et al., 2003; Di Consiglio et al., 2005; Abass et al., 2009).

As depicted in Fig. 5, by increasing the concentration of exposure, the inhibition of other P450 isoforms became progressively more and more important (Mutch and Williams, 2006). As already discussed, according to the different levels of P450 expression and genetic polymorphism in human populations, the involved P450 isoforms and the magnitude of the interactions with OPs could be different from that observed in this study (Hodgson and Rose, 2007a; Sams et al., 2000).

3.6. Interactive inhibition by low-dosed toxicant mixtures

The combined effect of the 4 OPs on P450 inhibition was finally assessed for the most affected microsomal isoform, namely, 2B6. Because humans may be exposed to a mixture of these 4 OPs (*e.g.*, through dietary exposure in drinking water, food, *etc.*) at relatively low concentrations, a specific approach to test chronic toxicity at the enzymatic level was considered (Fenik et al., 2011). The latter is based on the no-observed-effect-concentration (NOEC) and IC_{25} investigations and was adapted as a basis for further experiments for our *in vitro* P450 inhibition study (Diamond et al., 2013; Hasenbein et al., 2015).

NOEC was established as the concentration corresponding to 90% of the P450 2B6 remaining activity while the IC_{25} represented the concentration when an inhibition level of 25% of the enzyme activity is obtained. From the best fit for inhibition curves, NOEC and IC_{25} values

Table 2
CYP isoforms, probe substrates, model inhibitors, IC_{50} with their interval confidence (95%) for single substrate incubation and substrate cocktail incubation, p values and IC_{50} from the literature.

P450	Probe substrate	Model inhibitor	Assay	IC_{50} [μM]	*95% interval confidence* [μM]	F-test ($p = 0.05$)	IC_{50} literature [μM]
1A2	Phenacetin	α-Naphthoflavone	Single	0.031	0.023–0.042	$p = 0.91$	0.01–0.5[a,b,c]
			Cocktail	0.032	0.022–0.045		
2A6	Coumarin	Methoxsalen	Single	0.06	0.04–0.08	$^{*}p < 0.05$	0.14–2.11[c,d]
			Cocktail	0.16	0.14–0.19		
2B6	Bupropion	ThioTEPA	Single	7.44	5.25–10.55	$p = 0.58$	1.75–21[e,f,g]
			Cocktail	10.50	8.42–13.10		
2C8	Amodiaquine	Quercetin	Single	1.72	1.44–2.04	$p = 0.11$	3.3–57.8[c,h,i,j,k,l,m]
			Cocktail	1.45	1.25–1.69		
2C9	Diclofenac	Sulfaphenazole	Single	0.49	0.35–0.67	$p = 0.30$	0.05–1.5[e,l,m,n]
			Cocktail	0.59	0.48–0.73		
2C19	Omeprazole	(*S*)-Benzylnirvanol	Single	0.66	0.49–0.88	$p = 0.70$	0.098–0.41[c,e,o]
			Cocktail	0.72	0.47–1.12		
2D6	Dextromethorphan	Quinidine	Single	0.057	0.044–0.072	$p = 0.91$	0.009–0.68[a,e,n]
			Cocktail	0.055	0.036–0.082		
2E1	Chlorzoxazone	4-Methylpyrazole	Single	1.23	0.71–2.11	$p = 0.98$	0.87–15[a,f,p,q]
			Cocktail	1.11	0.62–1.99		
2J2	Astemizole	Flunarizine	Single	10.45	7.07–15.45	$p = 0.91$	0.94[r]
			Cocktail	11.68	7.62–17.59		
3A subfamily	Midazolam	Ketoconazole	Single	0.084	0.055–0.130	$p = 0.22$	0.007–0.1[a,e,h,l,s]
			Cocktail	0.062	0.046–0.084		
	Testosterone	Ketoconazole	Single	0.091	0.069–0.123	$p = 0.11$	0.005–0.15[a,e,l,m,s]
			Cocktail	0.067	0.049–0.091		

[a] From Caggiano and Blight (2013).
[b] From Weaver et al. (2003).
[c] From Kim et al. (2005).
[d] From Moreno-Farre et al. (2007).
[e] From Kozakai et al. (2012).
[f] From Khojasteh et al. (2011).
[g] From Turpeinen et al. (2005).
[h] From Dierks et al. (2001).
[i] From Walsky and Obach (2004).
[j] From O'Donnell et al. (2007).
[k] From Unger and Frank (2004).
[l] From Turpeinen et al. (2004).
[m] From Cai et al. (2004).
[n] From Dinger et al. (2014b).
[o] From Walsky and Obach (2003).
[p] From Bu et al. (2001).
[q] From He et al. (2007).
[r] From Ren et al. (2013).
[s] From Patki et al. (2003).

were extrapolated for each of the OPs and are as follows: fenitrothion 0.01/0.04 μM, methylparathion 0.02/0.06 μM, chlorpyrifos 0.09/0.2 μM and profenofos 0.5/1.6 μM. As depicted in Fig. 6, a mixture of OPs incubated at the NOEC level exhibited an inhibition potency similar to each individual IC_{25}. This demonstrated that exposure to a low-dose mixture of OPs could lead to a 25% decrease in the P450 2B6 metabolic capacity. Moreover, when incubated at the IC_{25} level, the mixture of OPs demonstrated a similar IC_{50} inhibition potency for P450 2B6, which mimicked an acute exposure to individual OPs. Therefore, the clinical relevance of chronic exposure to low concentrations of OPs mixtures coming naturally from environment could not be excluded, particularly for medical treatment involving P450 2B6 (Gerber et al., 2004; Hodgson and Rose, 2007b). In summary, when acting on the same P450, a simple cumulative effect was observed in the case of a mixture, suggesting that a similar mechanism of inhibition is likely involved. Other non-related pesticide combinations should be considered for evaluation if synergistic inhibition of one or multiple isoforms can be observed *in vitro* (Cedergreen, 2014).

The *in vivo* extrapolation of the observed OP inhibitions remains difficult to establish due to the ethical prohibition to perform clinical trials in the context of pesticide exposure. Moreover, *in vitro* inhibitions are often observed at concentrations that do not reflect *in vivo* levels. According to the basic static model for *in vivo* extrapolation, which relies on the few available *in vivo* data about the plasma concentration of the selected OPs to the *in vitro* apparent inhibition constant K_i (calculable from the IC_{50} value using the Cheng-Prusoff equation), the observed drug-toxicant interactions seem to be improbable giving that the systemic levels of OPs are globally <100 nM in controlled-exposure populations (Cheng and Prusoff, 1973; Meaklim et al., 2003). However, these interactions, particularly with P450 2B6, cannot be excluded in agricultural communities and in patients with mild-intoxication, in which much higher blood levels of OPs have been described due to prolonged and acute exposure (Huen et al., 2012). More comprehensive and accurate *in vitro-in vivo* extrapolations are possible by combining *in vitro*-derived pesticide specific parameters with physiologically based biokinetic (PBBK) models (*e.g.*, SimCYP) to predict the *in vivo* absorption, distribution, metabolism and excretion of pesticides (Foxenberg et al., 2011; Wilk-Zasadna et al., 2014). Moreover, by including *in vitro* P450-inhibition screening parameters into an average patient PBBK model, the developed cocktail approach will finally provide a high-throughput strategy for assessing the risks of a high number of toxicants at the population level.

4. Conclusion

The presented cocktail approach included, for the first time, eleven probe substrates for direct reversible inhibition screening of xenobiotics affecting the ten most relevant P450 isoforms in human metabolism. Thanks to the high analytical sensitivity, it was possible to minimize probe-probe interactions in the cocktail and drastically reduce both

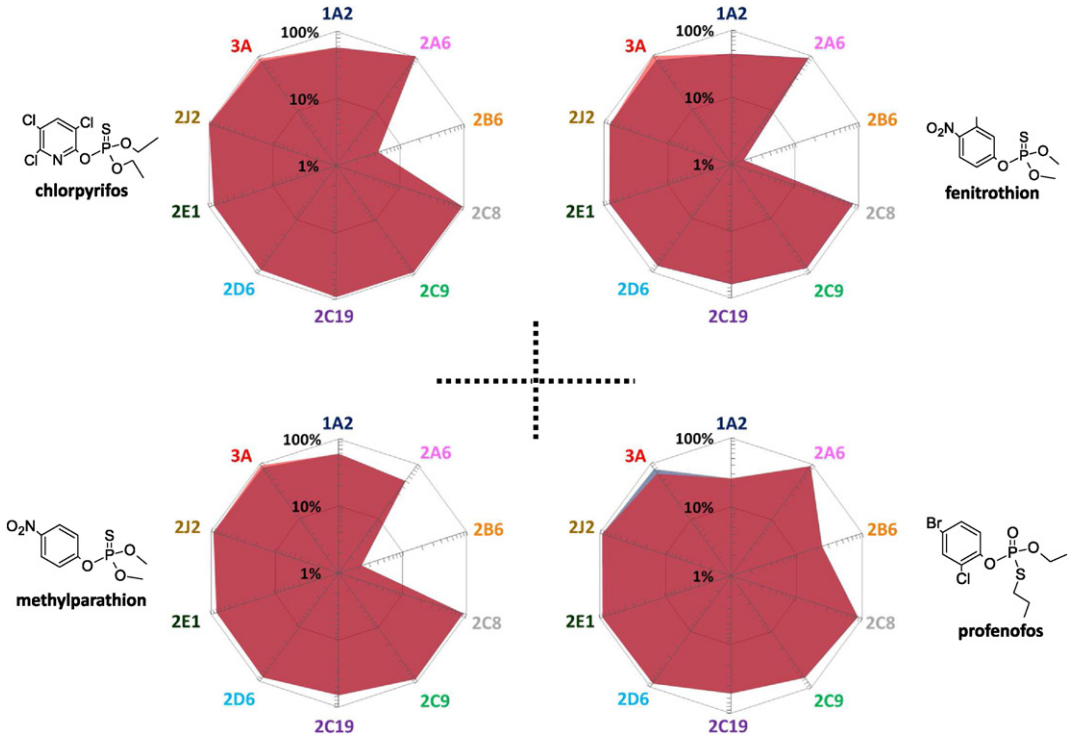

Fig. 4. Inhibitions of microsomal P450 isoforms by chlorpyrifos, fenitrothion, methylparathion and profenofos at a concentration of 10 μM.

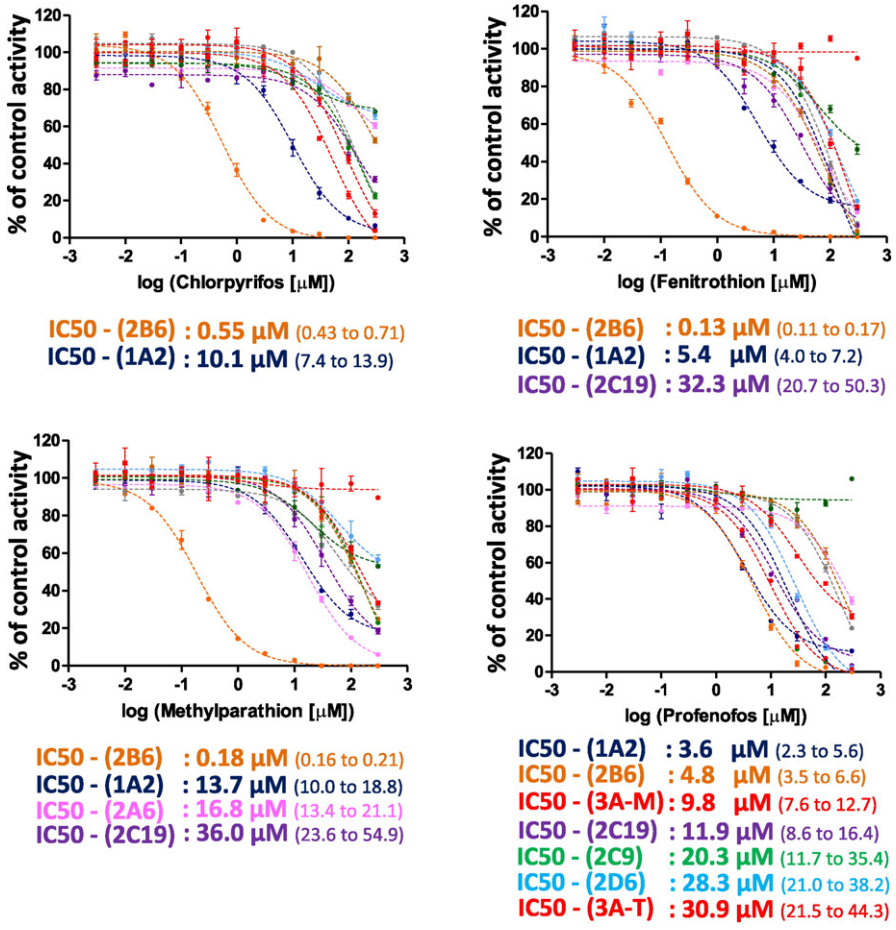

Fig. 5. Inhibition curves obtained with the cocktail approach. IC_{50} values are represented with interval confidence (95%). (●) 1A2, (●) 2A6, (●) 2B6, (●) 2C8, (●) 2C9, (●) 2C19, (●) 2D6, (●) 2E1, (●) 2J2, (●) 3A-M (midazolam), (■) 3A-T (testosterone).

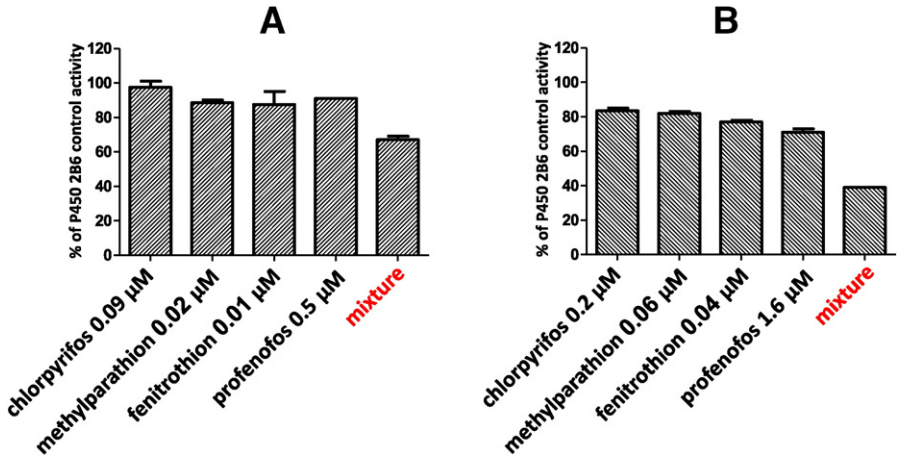

Fig. 6. Effects of the pesticides mixture incubated at the extrapolated NOEC (A) and IC_{25} (B) concentrations. Each bar represents the remaining P450 2B6 activity in presence of the inhibitor as a percentage (mean ± range, $n = 2$) of the metabolic ratio of the control incubation (solvent only).

the incubation time and reagent consumption. The IC_{50} values obtained were in agreement with reference values obtained by the single probe methods. A significant impact on various P450 activities was confirmed with organophosphorothionate insecticides. The P450 2B6 isoform was particularly affected, with IC_{50} values in the nanomolar range. Moreover, an additive effect was observed for pesticide mixtures incubated at very low concentrations, as expected for chronic environmental exposure. Hence, the developed cocktail approach represents a powerful tool for rapidly assessing direct reversible inhibition potential of individual as well as a combination of toxicants toward P450. The clinical relevance of these toxicological *in vitro* observations should be carefully interpreted due to the lack of reliable *in vivo* data establishing the link between the real environmental exposure and human systemic levels. However, as demonstrated for DDI, the reliable prediction of *in vivo* drug-toxicant interactions through an *in vitro*-based computational simulation could be possible in the near future.

Abbreviations

DDI	drug-drug interactions
DT	dwell time
ESI	electrospray ionization
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
pHLM	pooled human liver microsomes
LLOQ	lower limit of quantification
MeCN	acetonitrile
MeOH	methanol
Me_2SO	dimethyl sulfoxide
NADPH	β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt
NOEC	no observed effect concentration
OP	organophosphorothionate
P450	cytochrome P450
PBBK	physiologically-based biokinetics
QqQ/MS	triple-quadrupole mass spectrometer
S/N	signal-to-noise ratio
SRM	selected reaction monitoring
TIC	total ion current
UHPLC-MS/MS	ultra-high-pressure liquid chromatography-tandem mass spectrometry
ULOQ	upper limit of quantification

Data supporting the specificities of CYP-probe reactions and model inhibitors in the current incubation conditions are shown in Fig. S1. Analytical and metabolic variabilities of metabolic ratios during IC50 determination for single and cocktail approaches are shown in Table S1. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.taap.2016.04.013.

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Transparency document

The Transparency document associated with this article can be found, in the online version.

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