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Characterization of the main biotransformation pathways of synthetic cannabinoids from different chemical classes

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Abstract

The metabolic profile of synthetic cannabinoids from different chemical classes was characterized by means of *in vitro* approaches with human liver microsomes (HLM) and/or of cytochrome P450s (CYPs) and glucuronosyltarnsferase (UGT) isoforms. Results showed that for all investigated synthetic cannabinoids (SCs), regardless of the chemical class, mono-hydroxylation is the most important biotransformation pathway, followed by di-hydroxylation and, to a lesser extent, by dehydrogenation; instead, other metabolic reactions observed (i.e. dealkylation, dihydrodiol formation) appeared to be strictly dependent on the chemical nature of the specific substrate. Concerning the isoenzymes mainly involved in the phase I reactions, CYP3A4, CYP2D6 and CYP2C19 are the most accountable for mono-hydroxylation, di-hydroxylation and dihydrodiol formation abundant products, while CYP3A5 and CYP1A2 are the most involved in N-dealkylation and carbonyl formation. As for phase II metabolism, glucuronidation reactions for investigated substrates are mainly catalyzed by UGT2B7, UGT2B4, UGT1A3 and UGT1A10 isoforms.

Introduction

Synthesized in the early ‘90s in pharmaceutical research, SCs are not yet approved for human use, mostly because of their toxic side effects; nonetheless, they are illicitly marketed and widely abused as recreational drugs. At present, SCs are included in section S8 of the WADA Prohibited List, being their use prohibited “in competition” only [1,2]. Since SCs are extensively biotransformed [3,4], a deep knowledge of their metabolism is needed to identify proper administration marker(s).
Illegally marketed SCs are classified in (i) aminoalkylindoles (naphthoylindoles, phenylacetylindoles, benzoylindoles, naphthylmethylindoles), (ii) aminoalkylpirroles, (iii) naphtylmethylindenes, (iv) cyclohexylphenol and (v) classic cannabinoids [5]. For several, *in vivo* and/or *in vitro* metabolism was investigated, providing the following notable results:

1. Mono-hydroxylation at different positions and dihydrodiols formation are the most important biotransformation pathways for most of SCs [6-8];
2. carboxylation reactions occur for several alkylaminoindoles and phenylacethylindoles investigated [9-11];
3. carbonyl formation takes place mostly in phenylacethylindoles (JWH-250, JWH-203, JWH-251), classic cannabinoid HU-210 and cyclohexylphenol CP-47 [12-13].

Here, the *in vitro* phase I metabolism of SCs from different chemical classes has been evaluated in HLM to identify their biotransformation pathways; subsequently, for each pathway, the involvement of single CYP isoforms was evaluated to identify the best administration marker(s) in every condition; finally, for two SCs metabolites available as reference material, the contribution of different UGT isoforms in glucuronidation was evaluated.

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Experimental

Phase I metabolism substrates:
naphthoylindoles: JWH-018, JWH-073, JWH-007, JWH-210, JWH-122, WIN-55 212-2;
phenylacetylindoles: JWH-250;
naphthylmethylindoles: JWH-175;
classic cannabinoids: HU-210.
Phase II metabolism substrates:
naphthoylindoles: JWH 018 5-hydroxypentyl metabolite, JWH 073 N-5-carboxy metabolite.

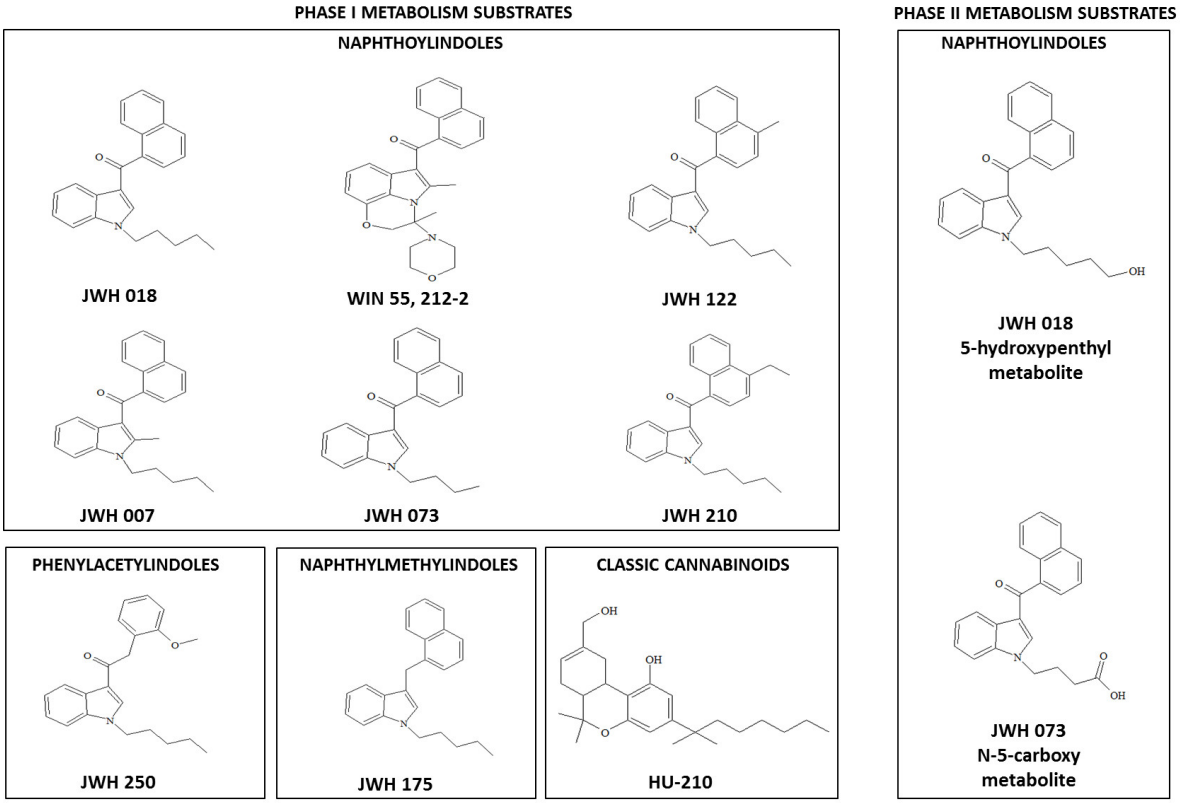

Fig. 1. Chemical structures of synthetic cannabinoids and their metabolites selected as phase I and phase II metabolism substrates.

In vitro assays

Substrates (Fig.1) were separately incubated at a final concentration of 10 µM; incubation mixtures (volume 250 µL) were set as follows:
Phase I metabolism: 0.1 M sodium phosphate buffer (pH 7.4), 3.3 mM magnesium chloride, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate and 0.4 UmL^{-1} glucose-6-phosphate dehydrogenase;
Phase II metabolism (glucuronidation): 0.1 M sodium phosphate buffer (pH 7.4), 8 mM magnesium chloride, 25 µg/mL alamethicin, 2mM cofactor uridine 5'-diphospho-α-D-glucuronic acid.
After mixture pre-warming (37°C, 5 minutes) the reactions were started adding (final concentration 0.5 mg/mL) HLM or c-DNA expressed CYP450 isoforms for phase I metabolism assays and HLM or c-DNA expressed UGT isoforms for phase II assays. Samples without enzymatic proteins (negative controls) were also prepared to monitor the potential non-enzymatic reactions. After incubation at 37°C for 4 hours, reactions were terminated by adding 250 µL of ice-cold acetonitrile; then, samples were cooled and centrifuged (12000 rpm, 10 minutes), the supernatants were prepared for instrumental analysis. All incubations were performed in triplicate.

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Sample pretreatment
Liquid/liquid extraction was performed for sample preparation; while phase I metabolism studies included a single direct extraction, samples from phase II assays underwent also a second extraction process after enzymatic hydrolysis to separate the free and glucuronidated fractions.
Initially, supernatants from all *in vitro* assays underwent liquid/liquid extraction carried out with 7 mL of tert-butylmethyl ether at pH 7.4 (phosphate buffer, 1 M) after addition of the internal standard JWH015-d_5 (final concentration 20 ng/mL); the organic and aqueous layers were separated and the former was evaporated to dryness and reconstituted in 50 µL of mobile phase. Aqueous layers from glucuronidation assays were added again with internal standard and underwent enzymatic hydrolysis (1 hour at 55°C) with β-glucuronidase from *Escherichia Coli;* after that, the second liquid/liquid extraction was carried out (same conditions reported above) to obtain the drug glucuronidated fraction. Finally, for each reconstituted extracted, aliquots of 10 µL were injected into the LC-ESI-MS/MS system.

Instrumental conditions
Samples were analyzed using an LC-MS/MS system; an Agilent 1200 series rapid resolution LC equipped with a Supelco Discovery C18 column (2.1x150 mm, 5 µm) operating in gradient elution at 250 µL/min (eluent A: water containing 0.1% (v/v) formic acid, eluent B: acetonitrile containing 0.1% (v/v) formic acid) was adopted. Mass spectrometry experiments were carried out on an API4000 triple-quadrupole instrument with electrospray ion source in positive mode at 500°C. Tandem mass spectrometry in product ion scan and selected reaction monitoring (SRM) acquisition modes were employed; in particular, for phase I metabolism samples, a specific SRM method was developed for each substrates, considering its theoretically possible metabolites; whereas for phase II samples, the SRM analytical method currently adopted in antidoping laboratory in Rome was used.

Results and Discussion

Phase I metabolism
In order to assist the detection and structure elucidation attempt of the *in vitro* generated metabolites, the fragmentation pattern of protonated selected substrates was defined by acquiring their product ion spectra after collision induced dissociation, at two collision energies (30, 50 eV); for all, the protonated molecule undergoes an extensive fragmentation, yielding to specific characteristic product ions.
In particular, for the aminoalkylindoles, product ions characteristic for the two main molecule portions were generated: characteristic ions at *m/z* 155 for JWH 018, JWH 073, JWH 007 and WIN-55 212-2, at *m/z* 183 for JWH 210, at *m/z* 169 for JWH 122, at *m/z* 141 for JWH 175 and at *m/z* 121 for JWH 250, were detected, representative for the respective naphthyl or methoxybenzyl moieties; signals related to the alkylindole moiety were recorded at *m/z* 214 for JWH 018, JWH 122, JWH 210 and JWH 250, at *m/z* 200 for JWH 073 and JWH 175, and at *m/z* 228 for JWH 007; for WIN-55 212-2 an abundant signal at *m/z* 100 was recorded for the morpholine group. Concerning HU-210, loss of water and the alkyl side chain generated abundant characteristic product ions at *m/z* 369 and *m/z* 261, respectively. As example, fragmentation pattern of JWH 175 is reported in Fig. 2.

A targeted analytical approach was followed for *in vitro* formed metabolite detection; on the basis of the protonated substrates fragmentation patterns and the bibliographic information concerning the hepatic metabolism of SCs, for the selected substrates all the theoretically possible metabolites and their possible fragments were postulated; an SRM method was developed with hypothesized metabolite specific transitions and finally their presence in incubated samples was checked. As example, a full chromatogram of JWH 210 detected metabolites is reported in Fig. 3.

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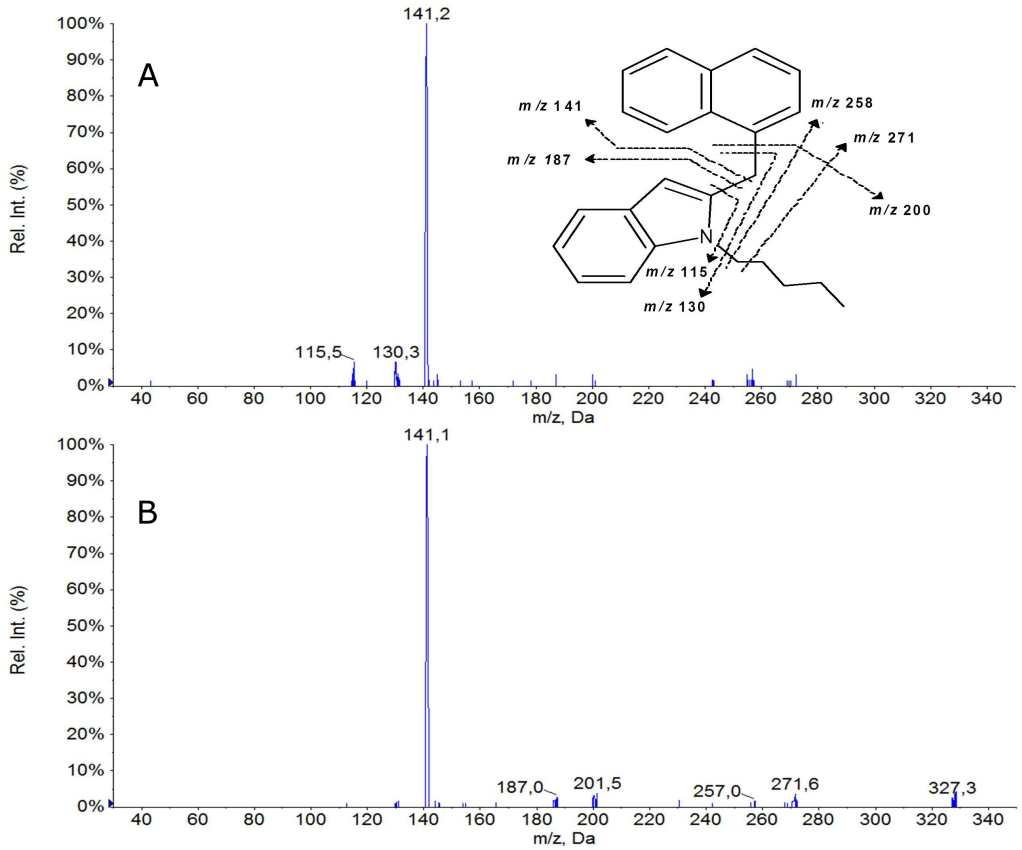

Fig.2: Fragmentation pattern of protonated synthetic cannabinoid JWH 175 (m/z 328) at 50 eV (A) and 30 eV (B).

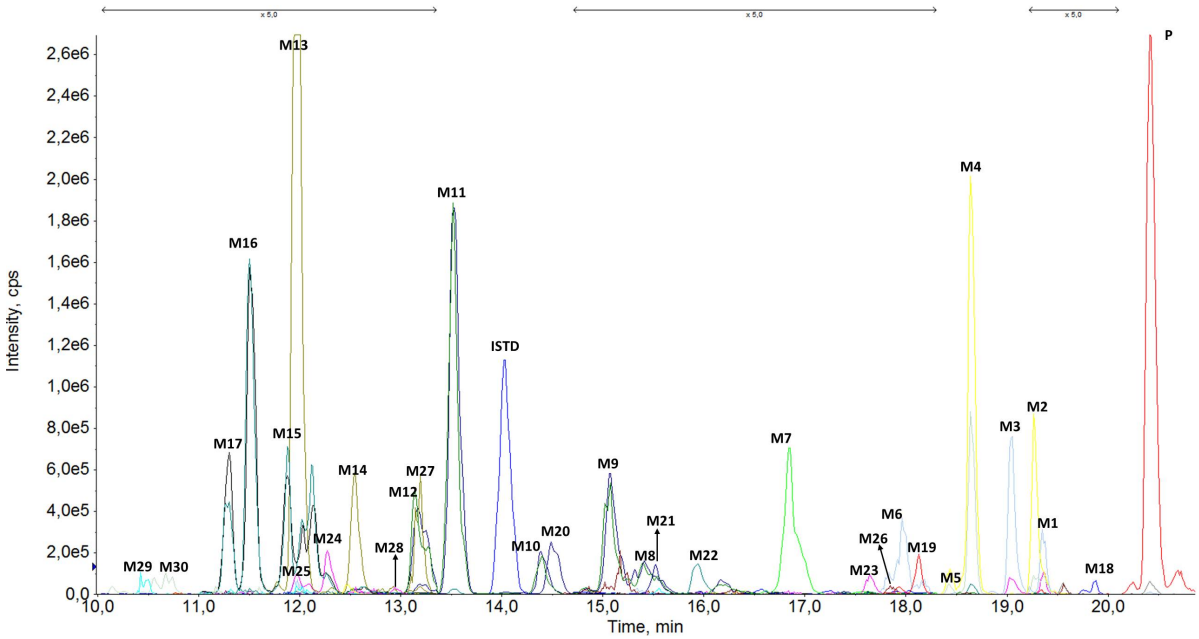

Fig.3. Chromatogram with all JWH-210 detected metabolites after in vitro incubation with HLM. P: JWH-210, M1-M6: monohydroxylated, M7-M12: dihydroxylated, M13-M17: trihydroxylated, M18: dehydrated, M19: dehydrated-monohydroxylated, M20-M21: dehydrated-dihydroxylated, M22-M23: dihydrodiols, M24-M25: dihydrodiols-monohydroxylated, M26: N-dealkylated, M27-M28: N-dealkylated-monohydroxy, M29-M30: dihydrodiols-dihydroxylated metabolites.

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In accordance with literature [6,7,9-11,13], obtained data showed all investigated substrates to undergo an extensive phase I metabolism in HLM (Tab.1); several biotransformation pathways were outlined and in many instances the moiety of the analyte carrying the metabolic modification was identified.
For all substrates, regardless of their chemical class, mono-hydroxylation resulted to be the main biotransformation route, being the alkyl side chain hydroxylated metabolites the most abundant detected. Products of di-hydroxylation at different locations were also detected, even though in lower amounts than mono-hydroxylation. Moreover, for all investigated SCs, significant amounts of products of alkyl-chain dehydrogenation, as such or combined with mono-hydroxylation, were detected. Finally, products of carboxylation at the alkyl-chain were detected for JWH 018, JWH 073, JWH 007, JWH 122, JWH 175 and HU 210.

	Naphthoylindoles						Phenylacethyl indoles	Naphthylmethyl indoles	Classical cannabinoids
METABOLIC PATHWAY	**JWH 018**	**JWH 073**	**JWH 007**	**WIN 55,212-2**	**JWH 122**	**JWH 210**	**JWH 250**	**JWH 175**	**HU-210**
Monohydroxylation	****	***	****	***	****	****	***	****	***
Di-hydroxylation	**	*	***	***	***	***	**		**
Tri-hydroxylation	**	*	***	*	**	***	*	**	---
Dehydrogenation	**	**	**	**	**	****	*	**	*
Dehydrogenation-mono OH	*	*	***	*	**	***	*	***	*
Dehydrogenation-di OH	*	**	*	*	*	***	---	*	---
Dihydrodiol formation	***	***	***	****	***	***	---	****	---
Dhydrodiol-mono OH	***	***	***	**	**	**	---	***	---
N-dealkylation	**	**	**	*	**	**	*	*	---
N-dealkylation-mono OH	*	**	*	*	**	**	*	*	---
Carboxylation	*	*	*	---	*	---	---	*	*
N-deakylation-dihydrodiol formation	*	*	**	---	*	---	---	---	---
Dihydrodiol-di-OH	---	---	*	---	---	*	---	*	---
Carbonyl formation	---	---	----	---	---	---	---	**	**
Carbonyl formation-mono OH	---	---	---	---	---	---	*	*	**

Tab.1: Phase I metabolic pathways detected for investigated synthetic cannabinoids after in vitro incubation with HLM.

Other metabolic routes depending on the SCs specific chemical structures were observed. For SCs having a naphthyl ring, the dihydrodiol formation was observed as substantial biotransformation route, yielding in two isomer products; moreover, products of dihydrodiol formation combined with mono-hydroxylation were detected for these substrates; no trace of it was instead recorded for JWH 250 nor HU 210, lacking of a naphthyl substituent. All aminoalkylindole SCs underwent also N-dealkylation, as such or combined with mono-hydroxylation.
Additionally, for JWH 175 and HU 210 carbonyl formation at different positions was observed, alone or combined with mono-hydroxylation, yielding in several isomer products; notable is the observation, never reported in literature before now, that carbonyl formation occurring on the methylene bridge of JWH 175 leads to the more potent synthetic cannabinoid JWH 018.

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Incubation sample using c-DNA expressed CYP450 isoforms permitted the identification of CYP3A4, CYP2D6, CYP2C19, CYP3A5 and CYP 1A2 as the most involved in phase I metabolism of the investigated substrates (Tab.2). In particular, reactions leading to the most abundant metabolites (i.e. mono- and di-hydroxylation, dihydrodiol formation) were mostly catalyzed by CYP3A4, CYP2D6 and CYP2C19, while CYP3A5 and CYP1A2 appeared to be the most accountable for N-dealkylation and carbonyl formation reactions.

	Naphthoylindoles						Phenylacethyl indoles	Naphthylmethyl indoles	Classical cannabinoids
METABOLIC PATHWAY	**JWH 018**	**JWH 073**	**JWH 007**	**WIN 55,212-2**	**JWH 122**	**JWH 210**	**JWH 250**	**JWH 175**	**HU-210**
Monohydroxylation	CYP 2D6	CYP 2C19	CYP 3A4	CYP 3A4	CYP 3A4	CYP 2D6	CYP 3A4	CYP 2D6	CYP 3A4
Di-hydroxylation	CYP 2D6	CYP 3A4	CYP 2D6	CYP 3A4	CYP2D6	CYP 3A4	CYP 2C19	CYP 2C19	
Tri-hydroxylation	CYP 3A5	CYP 2D6	CYP 3A4	CYP 3A4	CYP2D6	CYP 3A4	CYP 3A4	CYP 3A4	CYP 3A4
Dehydrogenation	CYP 3A5	CYP 3A4	CYP 3A5	CYP 3A4	CYP 3A4	CYP 3A4	CYP 3A5	CYP 3A4	---
Dehydrogenation-mono OH	CYP 2D6	CYP 3A5	CYP 3A4	CYP 3A5	CYP 3A4	CYP 3A4	CYP 3A4	CYP 2C19	CYP 3A4
Dehydrogenation-di OH	CYP 2C19	CYP 3A4	CYP 2C19	CYP 2C19	CYP 3A4	CYP 2C19	---	CYP 3A4	CYP 3A4
Dihydrodiol formation	CYP 2C19	CYP 2D6	CYP 2C19	CYP 2D6	CYP 2C19	CYP 3A5	---	CYP 3A4	---
Dhydrodiol-mono OH	CYP 2C19	CYP 2C19	CYP 3A5	CYP 2C19	CYP 3A4	CYP 3A5	---	CYP 3A5	---
N-dealkylation	CYP 3A5	CYP 3A5	CYP 1A2	CYP 1A2	CYP 1A2	CYP 1A2	CYP 3A5	CYP 1A2	---
N-dealkylation-mono OH	CYP 3A5	CYP 2C19	CYP 1A2	---	CYP 1A2	CYP 1A2	CYP 1A2	CYP 1A2	---
Carboxylation	---	---	CYP 1A2	---	---	---	---	---	CYP 3A4
N-deakylation-dihydrodiol formation	---	---	CYP 3A5	---	---	---	---	---	---
Dihydrodiol-di-OH	---	---	---	---	---	---	---	---	---
Carbonyl formation	---	---	---	---	---	---	---	CYP 3A5	CYP 3A5
Carbonyl formation-mono OH	---	---	---	---	---	---	---	---	CYP 3A4

Tab.2: Enzymatic isoforms of the cytochrome P450 most involved in the phase I biotransformation pathways of investigated synthetic cannabinoids.

However, since the catalytic activity of CYP3A4 and CYP2D6 may vary very significantly among different subjects (genetic polymorphisms, drugs co-administration, etc.) not only the most abundant mono-hydroxylated or dihydrodiol metabolites should be considered in identifying the best administration markers for investigated metabolites, but also N-dealkylation and carbonyl formation product, as their formation is less affected by external factors.

Phase II metabolism

JWH 018 5-hydroxypentyl metabolite and JWH 073 N-5-carboxy metabolite, available as reference standard materials, were incubated with HLM and c-DNA expressed UGT isoforms to investigate their glucuronidation profile and the enzymes most involved. After incubation and specific sample preparation free and conjugated fractions were separately analyzed in LC-MS/MS. As shown in Fig. 4, both of the selected metabolites undergo extensive *in vitro* glucuronidation, being almost completely absent in HLM conjugated fraction; UGT2B7, UGT2B4, UGT1A3, UGT1A4 and UGT1A1 resulted to be the most involved in glucuronidation of selected substrates; additionally, for JWH 073 N-5-carboxy metabolite also UGT 1A10 appeared to be discreetly involved. Obtained data are in agreement with bibliographic information [4].

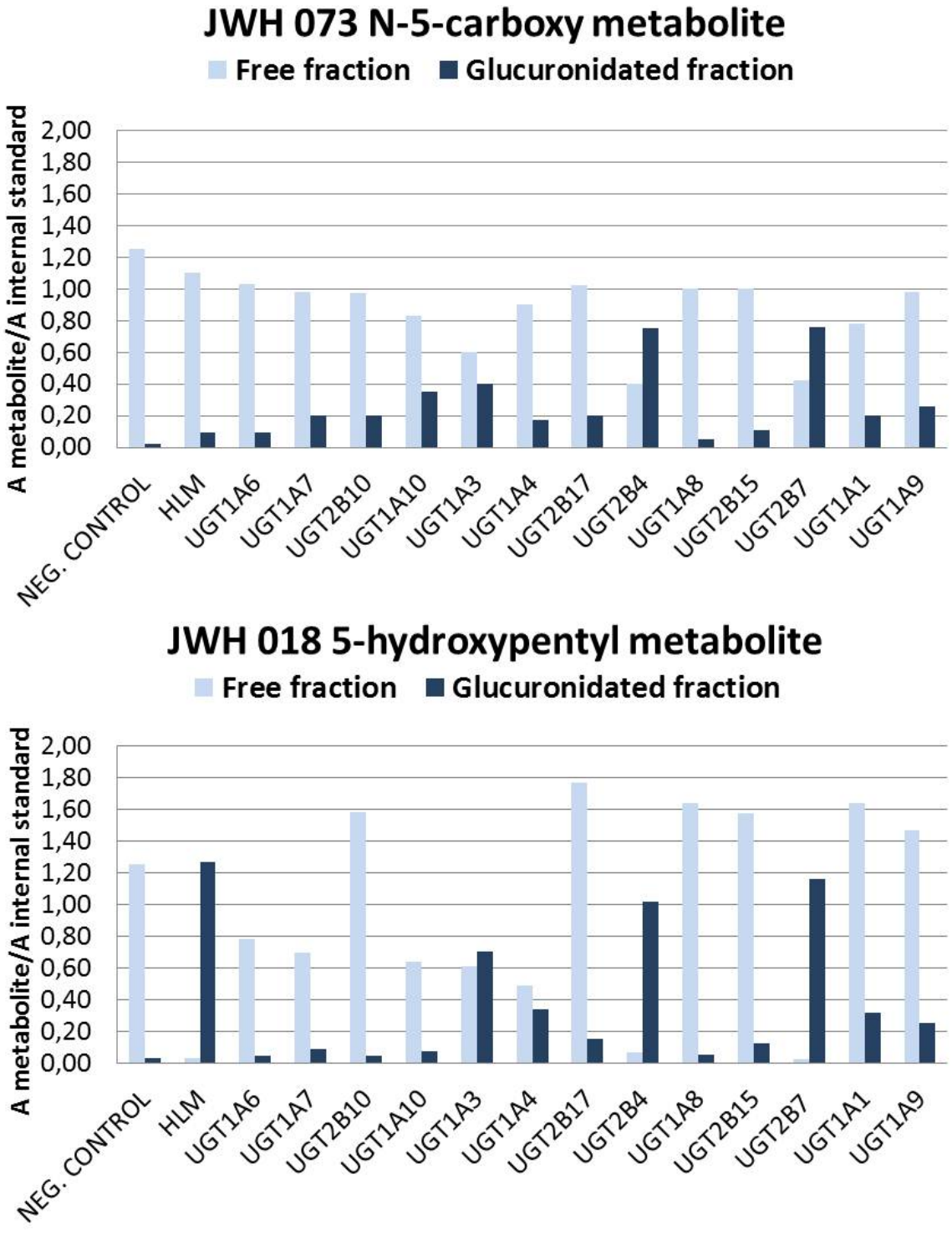

Fig. 4: Enzymatic isoforms of the uridine-diphosphate glucuronosyltransferase (UGT) system most involved in glucuronic acid conjugation of the selected substrates.

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Conclusions

The SCs investigated undergo extensive *in vitro* metabolism; the main phase I metabolic pathways are hydroxylation, dihydrodiol formation and dehydratation, in agreement with bibliography.
Enzymatic isoforms most involved in *in vitro* metabolism are the following:
► phase I: CYP3A4, CYP2D6, CYP2C19, CYP3A5 and CYP1A2;
► phase II: UGT2B7, UGT2B4, UGT1A3, UGT1A4 and UGT1A1.
Stated that the catalytic activity of several of these (i.e. CYP3A4, CYP2D6) may vary among subjects, evidence obtained in this study – to be confirmed by *in vivo* experiments – suggests that not only the most abundant mono-hydroxylated and dihydrodiol metabolites, but also N-dealkylated and/or carbonyl metabolites are the most proper and universal diagnostic markers of investigated SCs in antidoping analysis (Tab. 2).

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