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Elucidation of the Phase I and Phase II metabolic pathways of (±)-4'-methylmethcathinone (4-MMC) and (±)-4'-(trifluoromethyl) methcathinone (4-TFMMC) in cryopreserved human hepatocytes using LC-MS and LC-MS^2

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ABSTRACT:
The study investigates the in vitro metabolism of 4-MMC using cryopreserved human hepatocytes to characterize the associated Phase I and II metabolites. 4-MMC was incubated with human hepatocytes, and the reaction mixture was analyzed using a zwitterionic hydrophilic interaction (ZIC®-HILIC) column with LC-MS and LC-MS² techniques. The metabolism of 4-MMC resulted in the identification of 14 metabolites, which were structurally characterized based on accurate mass analyses and LC-MS² fragmentation patterns. The primary metabolic pathways for 4-MMC were identified as (i) oxidation of the 4'-methyl group and (ii) reduction of the β-keto moiety. Additionally, the biotransformation of a modified 4'-trifluoromethyl derivative (4-TFMMC) was studied, revealing significant differences in metabolism compared to 4-MMC. Notably, 4-TFMMC was found to be more extensively metabolized than mephedrone. Recent studies have indicated that the enzyme CYP450 (CYP2D6) plays a crucial role in the metabolic process, as it is responsible for Phase I metabolism. Consequently, the activities and expression of this drug-metabolizing enzyme were analyzed in the Life Technologies laboratory using cryopreserved human hepatocytes. A comparison of enzyme activities and expression from the two donors, alongside data from 196 other donors, revealed that hepatocytes obtained from these two donors exhibited intermediate CYP2D6 enzyme activity. Key pharmacokinetic parameters for both drugs were calculated, with biological half-lives (t½) found to be 693.0 minutes for 4-MMC and 216.6 minutes for 4-TFMMC. This data could contribute to a better understanding of in vivo metabolism in humans.

Keywords: 4-MMC, 4-TFMMC, Metabolism, HILIC, Mass Spectrometry.

1. INTRODUCTION:

Cryopreserved hepatocytes are a powerful tool for *in vitro* metabolic profiling of drugs. Human donor livers are only sometimes available, and cell cryopreservation technology has helped to solve this limitation of human hepatocytes availability. It provides a solution to many problems related to storage and distribution of freshly isolated human hepatocytes allowing conservation of viability as well as Phase I and Phase II metabolic activities. Cryopreserved freshly isolated cells can be processed easily by several protocols in which hepatocytes are suspended in a cryopreservation medium. The most common cryopreservation solutions are dimethyl sulfoxide (DMSO), propylene glycol, acetamide or polyethylene glycol. Cells are usually placed in a programmable freezer. Their viability of hepatocytes upon thawing decreases and may be below 55-65% but can be improved by using Percoll density gradient centrifugation to remove dead cells [1]. The main issue with using cryopreserved hepatocytes in drug metabolism studies is ensuring that the cells remain viable after thawing and being incubated in suspension at 37 °C. When comparing rat and human hepatocytes, it was observed that the viability of human cells post-cryopreservation was higher than that of rat cells under

the same conditions at 37 °C. Coundouris et al. found that human hepatocytes survive the cryopreservation process more effectively than rat liver cells when both are subjected to identical preservation conditions. [2]. Maintaining cell viability and cytochrome P45 levels is essential for ensuring the quality of cryopreserved hepatocytes needed for metabolism studies.[3]. Li et al. reported that cryopreserved human hepatocytes exhibited enzyme activities equivalent to those of freshly isolated human hepatocytes for CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, as well as for UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) [4]. In addition, Pedersen *et al*. reported that the cytochrome P450 2D6 (CYP2D6) gene is the major enzyme responsible for metabolising mephedrone during *in vitro* Phase I metabolism [5]. CYP2D6 is known to be a low-abundance enzyme in the liver, it plays a crucial role in the metabolism of many clinically used drugs. Approximately 20-25% of these medications are metabolized, at least in part, by this enzyme. Variations in the CYP2D6 gene can lead to different metaboliser types, including poor metabolisers (PM), intermediate metabolisers (IM), efficient metabolisers (EM), and ultra-rapid metabolisers (UM). [6].

Polymorphisms in the CYP2D6 gene can significantly influence the pharmacokinetics of approximately 50% of commonly prescribed medications. At standard doses, many drugs may lead to unexpected adverse reactions or fail to provide therapeutic effects. It has been suggested that these metabolic issues can arise from a deficiency in CYP2D6. Therefore, individuals who lack functional CYP2D6 genes may metabolise certain CYP2D6 substrates at a slower rate [6]. As a result, a decreased rate of drug biotransformation through metabolic pathways may enhance the risk of toxicity.

Many therapeutic classes of drugs are known to be metabolized by CYP2D6, which is involved in several metabolic pathways. For instance, CYP2D6 plays a role in the O-demethylation of codeine (an analgesic), encainide (an antiarrhythmic), and methoxyamphetamine (a recreational drug). It also participates in aromatic hydroxylation, which occurs in medications such as aprindine (an antiarrhythmic), desipramine (a tricyclic antidepressant), and tamoxifen (an antiestrogen). Additionally, CYP2D6 facilitates aliphatic hydroxylation, which affects drugs like perhexiline (a calcium antagonist), metoprolol (a beta-adrenergic blocker), and sparteine (an antiarrhythmic). Some studies have demonstrated the potential of CYP2D6 genotype to predict the risk of side effects and treatment outcomes.[7]. Spina and colleagues reported on a study involving thirty-one patients who were treated with oral desipramine at a dosage of 100 mg per day. After three weeks of treatment, it was found that two patients with the CYP2D6 poor metabolizer phenotype had the highest plasma concentrations of desipramine, a tricyclic antidepressant. These patients also experienced severe side effects [8]. This confirms the existence of a genotype that influences the metabolism of drugs processed by CYP2D6. Olesti and co-workers investigated the relationship between the CYP2D6 genotype and the incidence of acute toxicity episodes associated with mephedrone. Their data revealed that individuals with little or no CYP2D6 functionality were at a higher risk of experiencing acute toxicity when consuming mephedrone [9].

Meyer et al. conducted a study to identify metabolites of mephedrone and other designer drugs in the urine of rats and humans using the GC-MS technique. They detected six phase I metabolites of mephedrone in the urine of rats treated with doses comparable to those associated with amphetamine abuse. Additionally, seven metabolites were identified in human urine samples submitted for drug testing. [10]

Olesti et al. demonstrated that the concentrations of mephedrone and its metabolites in vivo in humans were assessed using LC-MS/MS. Their method was validated in human plasma and urine and was also employed to evaluate the ability of mephedrone and nor-mephedrone to cross the blood-brain barrier in vivo in rats, as these were the main metabolites found in the brain [11].

Two mass spectrometry-based techniques for fingerprint analysis of mephedrone and its metabolites were evaluated. The findings showed that liquid chromatography-tandem mass spectrometry (LC-MS/MS) was up to six times more sensitive than paper spray-mass spectrometry (PS-MS). However, while PS-MS may not be as effective for quantitative analysis, it can still serve as a useful diagnostic tool for qualitative analysis [12].

In our previous article, the Orbitrap-based LC-MS was utilized to identify metabolites in rat hepatocyte incubations, successfully detecting and elucidating the structure of numerous mephedrone metabolites in these cells [13].

Thus, this study aimed to utilize the same method for profiling the metabolites of mephedrone in vitro after incubation with human hepatocytes. This required the development of a new incubation procedure for the cryopreserved human cells.

2. Expermintal:

All reagents were of commercial quality (Sigma-Aldrich, Gillingham, UK) and used without further purification. 4-MMC (3a) and 4-TFMMC (3b) compounds were prepared using the previously reported method in rat hepatocytes study [14, 15].

Cryopreserved suspensions of human hepatocytes were obtained from CellzDirect, USA, via Life Technologies. This study was conducted using hepatocytes obtained from two different donors (in duplicate). Thawing Medium was Cryopreserved Hepatocytes Recovery Medium (CHRM®) (50 mL) (Lot: PLN00268) (APS Cat: 70001) (Invitrogen Life

Technologies, Cat.: CM7000). Incubation Medium were Williams' Medium E (500mL) (Lot: 988827) (Cat. No. : A12176), and Cocktail B (Lot: 1079913) (Cat No.:A13448) (Invitrogen Life Technologies).
24 well suspension culture plates were purchased from Greiner Bio-one (Lot; E10060ME). Orbital Incubator S150 (Stuart Scientific, UK) was used for cell incubation and set at 37 ºC.
Liquid chromatography-mass spectrometry (LC-MS) data were acquired using a Finnigan Exactive Orbitrap instrument (ThermoFisher Corporation, Hemel Hempstead, UK). Sample analysis was carried out with positive ion ESI detection. The mass scanning range was 70–1200 m/z, while the capillary temperature was 250 ◦C, spray voltage was +4.5 kV and the sheath and auxiliary gas (nitrogen) flow rates were 45 and 15, respectively (units not specified by the manufacturer). The LC-MS system (controlled by Xcalibur Ver. 2.0, Thermo-Fisher Corporation, Hemel Hempstead, UK) was run in binary gradient mode with an injection volume of 10 L. Solvent A was aqueous formic acid (0.1%, v/v) and solvent B was formic acid (0.1%, v/v) in acetonitrile; the flow rate was 0.3 mL min^{-1}. A ZIC®-HILIC (150 mm × 4.6 mm i.d., particle size: 5 m) column fitted with a ZIC®-HILIC guard column (HiChrom Limited, Reading, UK) was used for all analyses. The gradient programme was as follows: 20% A (0 min)to 50% A (at 12 min), to 50% A (at 26 min), to 80% A (at 28 min), to 80% A (at 36 min), to 20% A (at 37 min) and finally to 20% B at 46 min.
LC–MS^2 spectra were obtained by using a Finnigan LTQ Orbitrap (Thermo-Fisher Corporation, Hemel Hempstead, UK) with the same source, control software and chromatographic conditions specified above. The selected precursor ions were fragmented with a CID voltage of 40 eV. Data processing was carried out using SIEVEVer. 1.2.1 (ThermoFisher, Hemel Hempstead UK), ToxID Ver. 1.2.1 (ThermoFisher, Hemel Hempstead UK) and MetWorks Ver. 1.3 (ThermoFisher, Hemel Hempstead UK) software

2.1. Method and incubation protocol:

transferring 10 µL of stock solutions (100 mM) into tubes, then making them up to 5mL with an incubation medium to prepare substrates at 200µM Cryopreservation medium (CHRM) was aliquoted into 50 mL tubes, and placed in a water bath to warm at 37 °C, and then two 50 mL tubes of complete incubation medium (Williams' E Medium and Cocktail B) were prepared and warmed for 15-20 minutes at 37 °C. vials of cryopreserved hepatocytes were immersed in a 37 ºC water bath and the contents were poured into 50 mL warmed thawing medium (CHRM) as soon as possible. The mixture (thawed medium with cells) was centrifuged at 100 g for 10 minutes, then the tube was removed from the centrifuge being careful not to disturb the pellet in the bottom of the conical tube, and the supernatant poured off completely.
The cell pellet in the bottom of the conical tube was gently resuspended by adding approximately 1-2 mL of warmed Williams' E medium. Then additional medium was added to give 2-6 mL. In this study, the volumes were 4.5 and 3.85 mL of the first and second batch of cells respectively.
The cell viability was confirmed using a Trypan blue (0.1%w/v) exclusion test. The initial viability was 87.15% (± 0.07; n=2) in HU4215 cell samples and 90.2% (± 1.56; n=2) in HU4226 cell samples. The suspensions were diluted to reach a concentration of approximately 10^6 cells mL^{-1} with 37 °C Williams' E medium.
Cells were incubated with 4-MMC (3a) and 4-TFMMC (3b) (100 µm) separately on a shaking table at 120 rpm, in an incubator at 37ºC with an atmosphere of air / 5% CO_2 in the clean room. Each drug incubation was sampled in duplicate at 0, 30, 60, 90 and 120 minute time points. As described above, each well contained 0.5 mL of suspension, consequently, from each time point 10 µL was taken from the incubation into an Eppendorf tube for a quick check on viability using Trypan Blue. Aliquots (0.20 mL) of incubation suspension were then removed from individual wells and placed in Eppendorf tubes labelled for the appropriate time points, and the reaction was quenched by adding 0.70 mL of acetonitrile. Thus, each well was used to take 2 time points, and samples were then frozen at - 80 ºC until analysis.

2.2. Effects of 4-MMC (3a) and 4-TFMMC (3b) on viability of cryopreserved human hepatocytes:

To examine the toxic effects of 4-MMC (3a) and 4-TFMMC (3b), samples were taken at regular time intervals (0, 30, 120 minutes). The viability of cryopreserved hepatocytes was measured using Trypan Blue exclusion and Table 22 shows the results expressed as the percentage of live cells. An aliquot (10 µL) of the cell suspension was added to (10µL) of Trypan Blue (0.1%w/v), the mixture was loaded onto a haemocytometer and the cells were counted under the microscope.

2.3 Sample Preparation for LC-MS:

The samples were thawed at room temperature and centrifuged at 104 rpm for 15 minutes. Any remaining protein was then removed by adding 0.1 mL of acetonitrile followed by filtration through a Biotage Isolute® PPT+ protein precipitation plate (Biotage Limited, Sweden) [16]. The filtrate was collected and analyzed by either LC-MS or LC-MS^2. The efficiency of recovery from hepatocyte matrix was determined to be 104.5 ± 3.8% (for 4-MMC (**3a)**, n = 4, ± SD), 100.7 ± 3.9% (for 4-TFMMC (**3b)**, n = 4, ± SD) respectively.

3. RESULTS AND DISCUSSION:

3.1. Effects of cryopreservation of human hepatocytes on drug-metabolizing enzymes:

Table 1 shows the activities of a range of Phase I and Phase II reactions measured in the hepatocytes isolated from the two donors, demonstrating that they are competent for the CYP-catalysed reactions, and for the conjugation pathways which were shown in previous rat hepatocytes studies take part in the metabolism of the mephedrone analogues being investigated.

Table 1: The activities of a range of phase I and phase II reactions measured in the hepatocytes isolated from the two donors

Lot No.	HU4215	HU4226
Medium Method	WEM[a]	WEM
Enzymes	Metabolic activities*	
7-HCG[b]	844.00	401.00
7-HCS[c]	65.80	38.00
CYP1A2	146.00	54.90
CYP2B6	233.00	20.80
CYP2C8	2.97	1.39
CYP2C9	72.50	58.80
CYP2C19	170.00	39.50
CYP2D6	21.10	8.64
CYP2E1	74.00	81.20
CYP3A4 - TEST	1,580.00	613.00
CYP3A4/5 - MDZ	634.00	160.00
FMO	114.00	311.00

[a] Wiliams' E medium.
[b] Glucuronidation of hydroxycoumarin.
[c] Sulphation of hydroxycoumarin.
*Metabolic activities are determined by HPLC or LC-MS/MS analysis and recorded as pmol/10^6 cells/min.

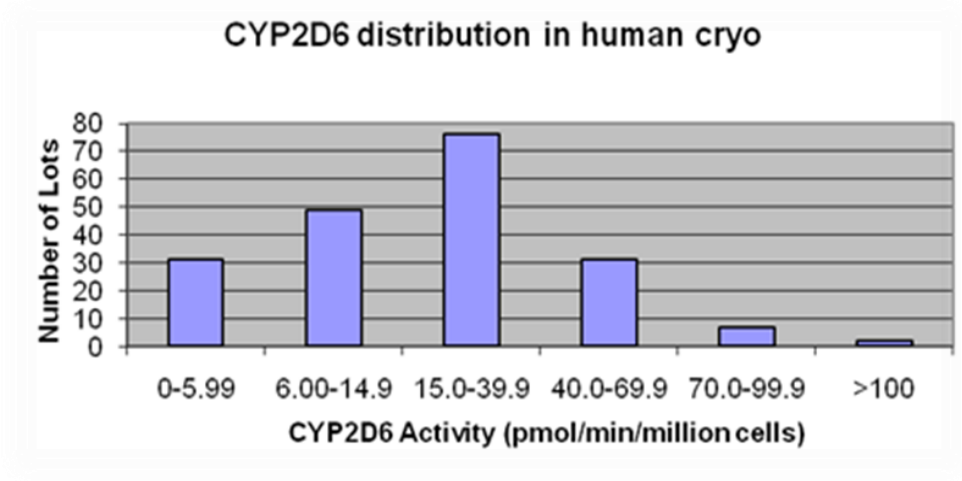
Phase I reactions of most current drugs are catalysed by five CYP enzymes CYP3A, CYP2D6, CYP2C9, CYP1A2, and CYP2E1 [17]. In this study, drug metabolizing enzymes were analysed in Life Technologies laboratory for activities and expression in cryopreserved human hepatocytes. This work was carried out in Drug Discovery ADME/Tox Division of Life Technologies, Durham NC USA, by Dr. Jonathan P. Jackson. The expression of 13 CYP isoform enzymes was determined in the human hepatocytes which were obtained from the two donors (HU4215 and HU4226) after cryopreservation.

Pedersen and co-workers showed that the activity of CYP2D6 contributed to the microsomal metabolism of mephedrone [5]. Both donors used in the current study were heterozygous for the CYP2D6*4 allele. This allele synthesises an enzyme devoid of activity (defective splice site), however, since these individuals were heterozygous their hepatocytes should have intermediate CYP2D6 activity (between a poor and effective metaboliser). Currently, more than 46 different major polymorphic CYP2D6 alleles are known [6], but in the current study, only 4 different CYP2D6 SNPs were tested for. Thus, the other alleles may be affected by a different SNP which was not tested. The other possible CYP2D6 defective alleles are CYP2D6*2, CYP2D6*10, CYP2D6*17, CYP2D6*41.

The activities of CYP2D6 (measured by the de-methylation of dextromethorphan) expressed as pmol/min per million cells for HU4215 and HU4226 were determined to be 21.1 and 8.64, respectively. Comparing CYP2D6 activity of these two donors to that of 196 other donors it appears that HU4215 and HU4226 fall at the median value and below. This comparison supports our genotyping data showing that the donors should have an intermediate CYP2D6 enzyme activity phenotype based upon the heterozygous genotype, and the activities are a little lower than expected.

In the distribution of the 196 donors compared to HU4215 and HU4226 shown in Figure 1, the median of the population was 19.6, the average was 24.7, and the data ranged from 0.2 to 118 pmol/min/10^6 cells.

Figure1: CYP2D6 activity of 196 donors



Additionally, both donors were homozygous null for CYP3A5*3 activity and HU4226 was a heterozygous CYP2C9*3 individual. The CYP2C9*3 allele produces a defective enzyme with reduced activity, but CYP2C9*3 is not devoid of activity.

3.2. Biotransformation of 4-MMC (3a) and 4-TFMMC (3b) in Cryopreserved human hepatocytes:

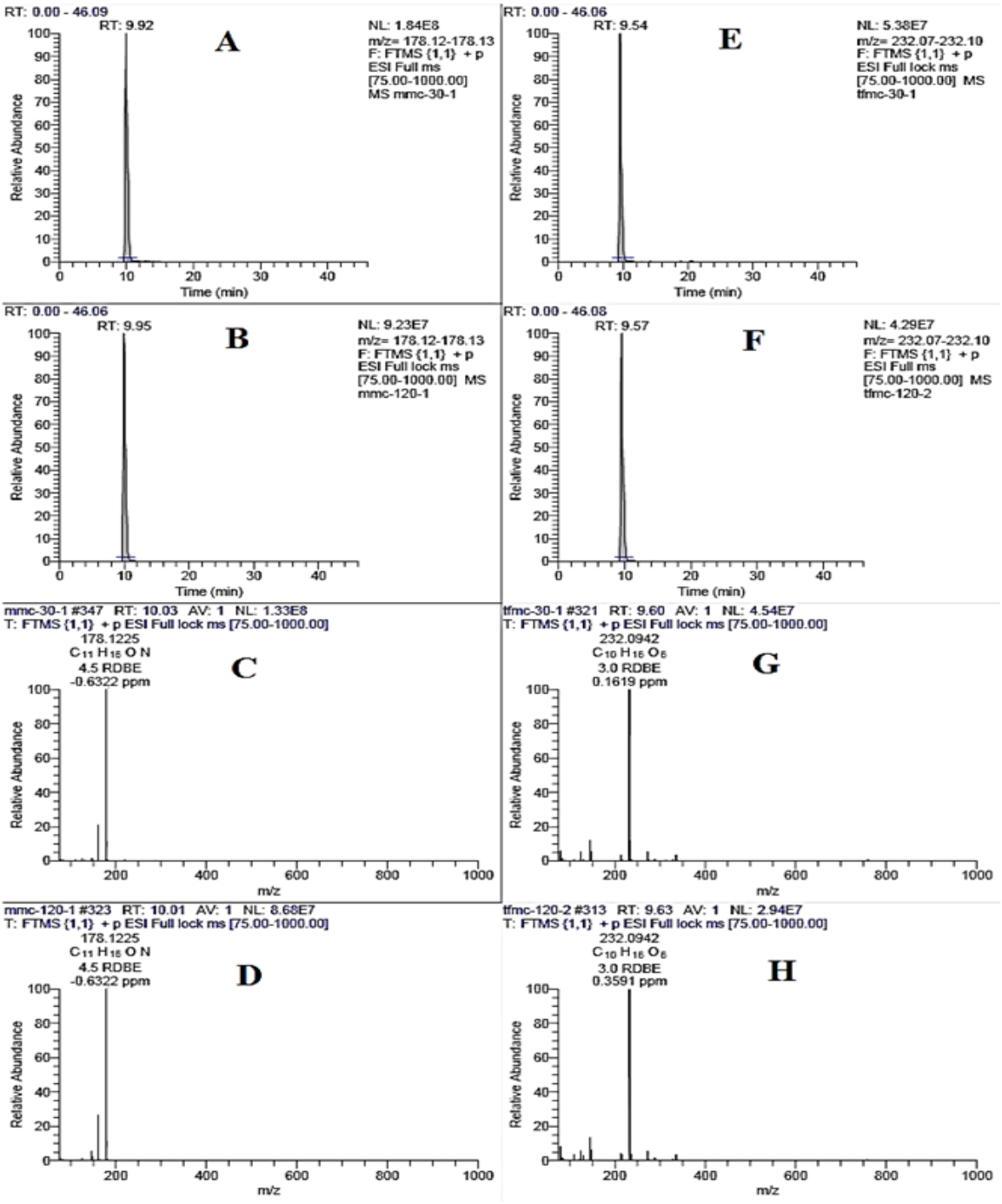
After 4-MMC (3a) and 4-TFMMC (3b) were incubated with human hepatocytes, samples were taken at various time points and compared to pre-incubated samples (time zero). The viability of cryopreserved hepatocytes was confirmed to be 81.3 ± 2.8% at the beginning of the experimental procedure and found that the metabolism of 4-MMC (3a) and 4-TFMMC (3b) had no significant effect on the viability of hepatocytes throughout the 2h incubation at a concentration of 100µM.

Metabolites to be identified were present in the incubated samples at both 30 minutes and after 120 minutes. However, some of the metabolites previously identified in the rat hepatocyte incubations were not present in incubations with human hepatocytes, particularly some of the Phase II metabolites. The metabolism rate was slower in human hepatocytes than in rat hepatocytes, and most of the metabolites were observed with lower abundance in human cells than in rat hepatocytes.

Figure 2 (A, B) and (E, F) shows selected ion chromatograms at 178.122 Da and 232.094 Da of 4-MMC (3a) and 4-TFMMC (3b) respectively. The chromatograms (A, E) represent [M+1] peaks at 30 minutes, while (B, F) show chromatograms for peaks after 120 minutes of incubation with human hepatocytes. Peak areas for the selected ion chromatograms indicated only a small change in the levels of parent drugs during the incubation. This is also reflected in the ESI spectra in Figure 2 (C, D) and (G, H) which show only a small difference in the relative intensity of the molecular ion peak between the 30-minute and 120-minute incubation periods.

LC-MS^2 experiments were conducted to support metabolite structural elucidation, and the proposed fragments of some 4-MMC (3a) and 4-TFMMC (3b) are presented in appendix (1).

Figure 2: Selected ion chromatograms from LC-MS analysis and mass spectra of 4-MMC (3a) and 4-TFMMC (3b). (A) and (B) selected ion chromatograms illustrate the M^+ ion at m/z =178, at 30 and 120 minutes respectively; (E) and (F) selected ion chromatograms illustrate the M^+ ion at m/z =232, at 30 and 120 minutes respectively;(C) and (D) the mass spectra of the 178 M^+ ion identify the 3a compound, at 30 and 120 minutes respectively ;(G) and (H) the mass spectra of the 232 M^+ ion identify the 3b compound, at 30 and 120minutes respectively.



Some of the Phase I metabolites of mephedrone (4-MMC, 3a), along with a few Phase II metabolites, such as glucuronides and acetylated forms, were detected at the end of the 120-minute incubation period, although in trace amounts. The parent drug and its metabolites are also presented in Table 2.

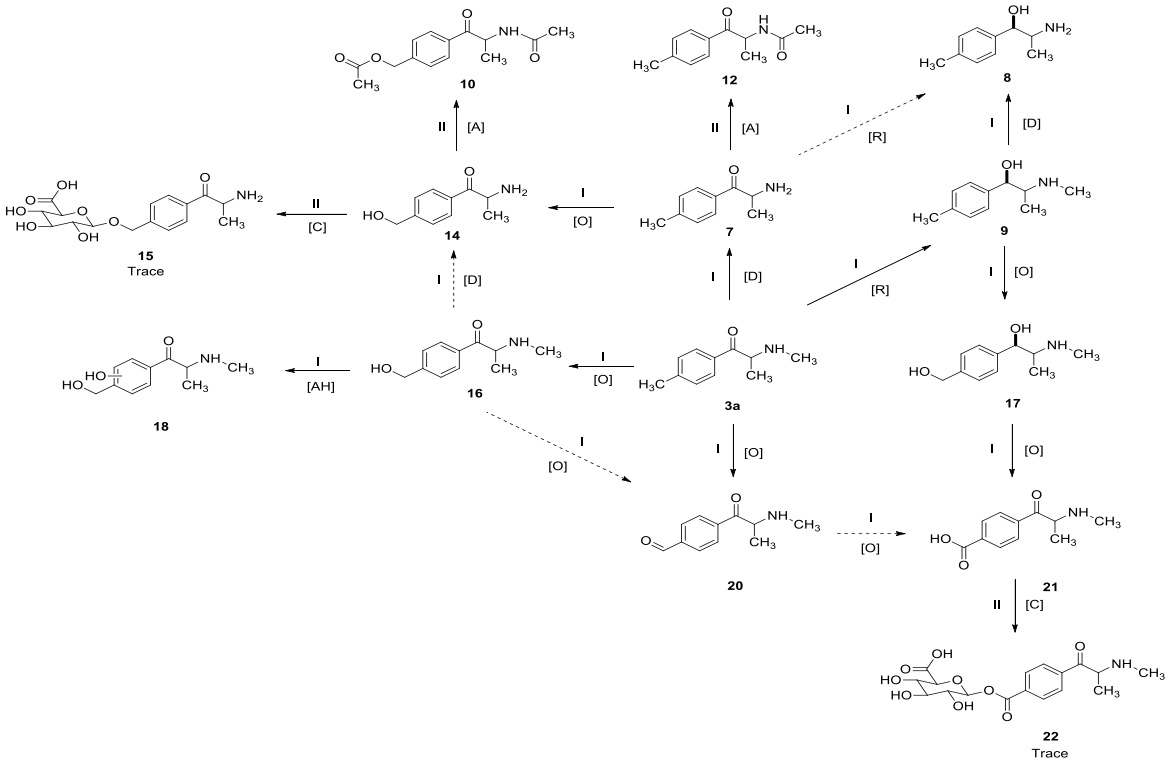
All metabolic pathways for 4-MMC (3a) illustrated in Scheme 1 were previously identified using freshly prepared rat hepatocytes. When comparing the metabolic profiles derived from rat and human hepatocytes, the same metabolic pathways were observed; however, there were notable differences in the abundance of most metabolites. The rate of metabolism was significantly higher in rat hepatocytes compared to human hepatocytes. Additionally, some metabolites were not present in the incubations with human hepatocytes, which will be discussed later.[18].

In this study, the major metabolites of 4-MMC (3a) were normephedrone (7, t_R = 10.4 min, *m/z* =164.1070), 4-hydroxy-methylmethcathinone (16, t_R = 8.3 min, *m/z*=194.1175). Also, an unidentified metabolite (U, *m/z* = 194.1176) was observed as a major metabolite, and it may be the result of hydroxylation of mephedrone on the aromatic ring rather than hydroxylation of 4-methyl group as in metabolite 16. Metabolite U differs from metabolite 16 in that metabolite U has a different chromatographic behaviour.

Table 2: LC-MS Exactive Orbitrap data for biotransformation of 4-MMC (3a) incubated with human hepatocytes (120 min). See Scheme 11 for proposed metabolite structures. Key: gluc = glucuronic acid-H2O; U = unidentified metabolite (tR = 8.3 min; m/z = 194.1176); ND = Not Detected. The mass errors of the detected metabolites were within ± 1- 2 ppm of their assigned elemental composition.

Met.	Rt (min)	m/z	Formula (ppm)	MS2 Base Peak	Other Ions (%)
12	6.0	206.1176	$C_{12}H_{16}NO_2$(0.044)	160	120(9)
20	6.0	192.1019	$C_{11}H_{14}NO_2$ (-0.037)	164	146(9),119(4)
10	6.1	264.1228	$C_{14}H_{18}NO_4$ (-0.53)	ND	
15	7.0	356.1335	$C_{16}H_{22}NO_8$ (-1.3)	ND	
U	8.3	194.1176	$C_{11}H_{16}NO_2$ (-0.0081)	ND	
14	9.0	180.1019	$C_{10}H_{14}NO_2$ (0.12)	162	144(2)
18	9.4	210.1125	$C_{11}H_{16}NO_3$ (-0.060)	135	192(17), 174(4.7), 162(13.6)
3a	9.9	178.1225	$C_{11}H_{16}NO$ (-0.63)	160	147(3)
9	10.2	180.1382	$C_{11}H_{18}NO$ (-0.68)	162	160(10)
7	10.4	164.107	$C_{10}H_{14}NO$ (0.024)	146	147(16)
8	10.6	166.1226	$C_{10}H_{16}NO$ (-0.13)	148	120(48)
21	11.1	208.0968	$C_{11}H_{14}NO_3$ (-0.16)	146	190(71),172(34)
16	11.5	194.1175	$C_{11}H_{16}NO_2$ (0.17)	146	176(12),158(51),133(5)
17	12.5	196.1332	$C_{11}H_{18}NO_2$ (0.098)	138	178(92), 148(2)
22	14.4	384.1288	$C_{17}H_{22}NO_9$ (-0.12)	ND	

Scheme 1: Proposed scheme for the Phase I and II metabolism of (±)-mephedrone (4-MMC, 3a) in cryopreserved human hepatocytes. Metabolite numbers correspond to the metabolite data presented in Table 25. Key: [A] = acetylation; [R] = reduction; [D] = demethylation; [O] = oxidation; [C] = conjugation; [AH] = aromatic hydroxylation (The blue rectangle highlights the most abundance metabolites).



Many of these fragmentation pattern interpretations are just putative. They fit with the elemental compositions obtained for the fragments but often do not fit the expected rules of fragmentation.

The proposed biotransformation of 4-TFMMC (3b) detected after incubation of human hepatocyte is summarised in Scheme 2, and supporting data obtained from some predominant metabolites obtained by using the LTQ Orbitrap in MS^2 mode are shown as proposed fragment structures in Table 3. Hydroxy (trifluoromethyl) methcathinone (23, t_R = 6.6 min, *m/z* = 248.0891), (±)-4'- (trifluoromethyl) ephedrine (31, t_R = 9.7 min, *m/z* = 234.1098), and 4'-(trifluoromethyl) cathinone (28, t_R = 9.8 min, *m/z* = 218.0787) were identified as major Phase I metabolites. Further steps of Phase I metabolism are shown in Scheme 2 and some conjugated Phase II metabolism occurs producing a trace amount of *O*-glucuronidation (30, t_R = 6.3,*m/z* = 410.1059) and *N*-acetylation (29, t_R = 5.7,*m/z* = 260.0892) metabolites. The dynamic range of the HRMS was sufficient to detect them.

As mentioned in rat hepatocytes study, the aromatic hydroxylation metabolite of 4-TFMMC(3b) can occur in the ring instead of the tolyl moiety which is blocked in this compound by CF_3, and the aromatic ring itself becomes a site of metabolism, and substitutes the hydrogen atom in the ring within an electrophilic substituent.

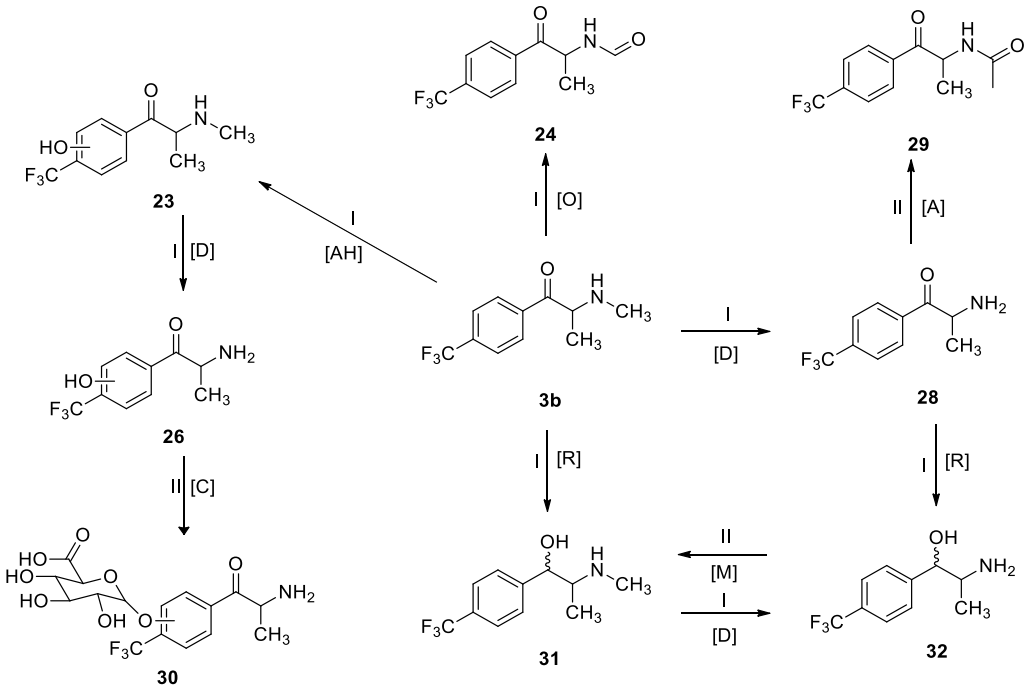
MS/MS fragments showed specific neutral loss occurring in ESI-MS for certain chemical moieties in 4-MMC (3a) and 4-TFMMC (3b). For example, a water loss (-18 Da) can be commonly observed for keto moiety and alcohol moiety in most metabolites. A neutral loss of CO (-28Da) can be observed from aldehyde moiety of metabolite such as (20, *m/z*=192.10) and (24, *m/z*=246.07), which cannot be observed in other metabolites, Tables 2 and 3 summarise some MS/MS ions for some metabolites.

Table 3: LC-MS Exactive Orbitrap data for biotransformation of 4-TFMMC (3b) incubated with human hepatocytes (120 min). See Scheme 12 for proposed metabolite structures. The mass errors of the detected metabolites were within ± 0.05 -1.0 ppm of their assigned elemental composition.

Met.	Rt (min)	m/z	Formula (ppm)	MS2 Base Peak	Other Ions
29	5.7	260.0892	$C_{12}H_{13}F_3NO_2$ (-0.22)	216	218(27)
24	5.8	246.0736	$C_{11}H_{11}F_3NO_2$ (-0.19)	218	173(17),200(29)
30	6.3	410.1059	$C_{16}H_{19}F_3NO_8$ (0.41)	ND	
23	6.6	248.0891	$C_{11}H_{13}F_3NO_2$ (-0.84)	173	230(45), 202(7),212(6)
26	7.5	234.0736	$C_{10}H_{11}F_3NO_2$ (-0.26)	ND	
3b	9.7	232.0942	$C_{11}H_{13}F_3NO$ (-0.79)	214	
31	9.7	234.1098	$C_{11}H_{15}F_3NO$ (-0.82)	201	216(15),214(2), 196(7),165(23)
28	9.8	218.0787	$C_{10}H_{11}F_3NO$ (0.047)	200	201(60),173(2.3)
32	9.8	220.0944	$C_{10}H_{13}F_3NO$ (0.28)	202	
*ACN-28	9.8	259.1051	$C_{12}H_{14}F_3N_2O$ (-0.78)	ND	

*ACN-28 = Adducts of Acetonitrile of metabolite (28)

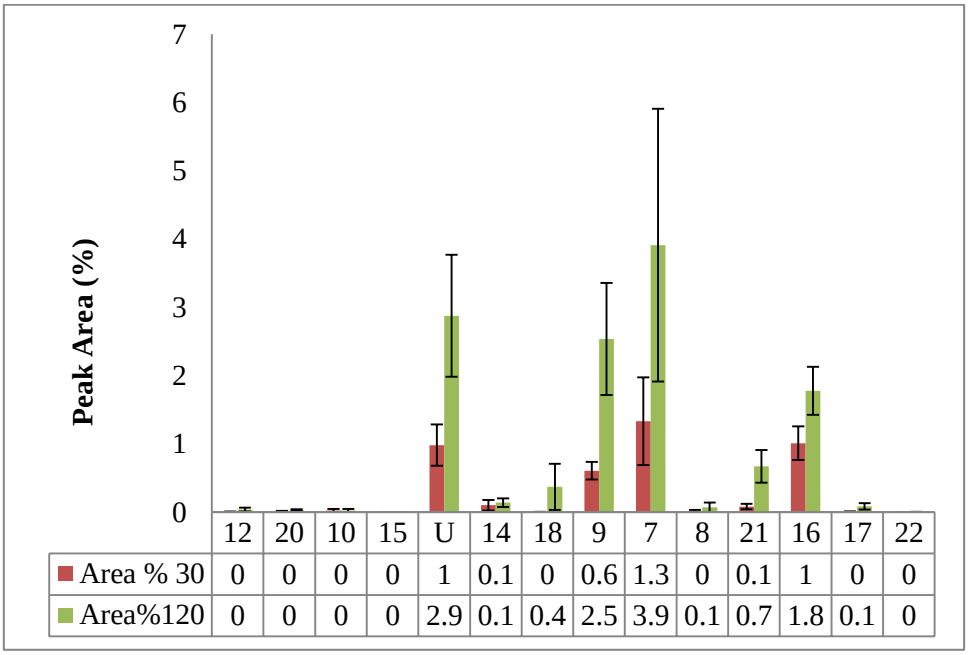
Scheme 2: Proposed scheme for the Phase I and II metabolism of (±)-4'-(trifluoromethyl) methcathinone (4-TFMMC, 3b) in cryopreserved human hepatocytes. Metabolite numbers correspond to the metabolite data presented in Table 26. Key: [A] = acetylation; [R] = reduction; [D] = demethylation; [O] = oxidation; [C] = conjugation; [AH] = aromatic hydroxylation; [M] = methylation (The blue rectangle highlights a most abundance metabolite).



3.3. Metabolic profiling of 4-MMC (3a) and 4-TFMMC (3b) in cryopreserved human hepatocytes:

The metabolic profiles of 4-MMC (3a) and 4-TFMMC (3b) were examined in cryopreserved human hepatocytes from the two donors. Figures 3 and 4 show that 4-TFMMC(3b) was more extensively metabolised than 4-MMC(3a), however, the rate of Phase I and II metabolism was slow for both drugs, and the most abundant chemical detected in the incubations was the parent drugs. The histogram (Figure 3) of 4-MMC (3a) indicates that normephedrone (7, *m/z* =164.1069) was formed in larger amounts than the other metabolites detected, and it increased after two hours incubation period by approximately 3%. The hydroxylated metabolites (U, *m/z* =194.1175) (9, *m/z* =180.1383) were also increased over the two hour incubation period by approximately 2%. The major compound detected was mephedrone itself. Very low levels of glucuronidation and acetylation biotransformation could also be observed. There were two trace amounts of glucuronide metabolites that could be detected in one hepatocyte sample (donor HU4215), and these were not present in HU4226 incubation. A possible explanation for this might be that it was probably due to differences in enzyme activities between these two donors. In addition, the results of this study indicate that incubation of samples from the donor (HU4215) produces a higher abundance of Phase I metabolites than the donor (HU4226). Particularly, the results found that donor (HU4215) produced a relatively higher abundance of hydroxylated and dealkylated metabolites such as (7, 16, U) in comparison with donor HU4226 and this may account for the production of more Phase II metabolites such as glucuronides.

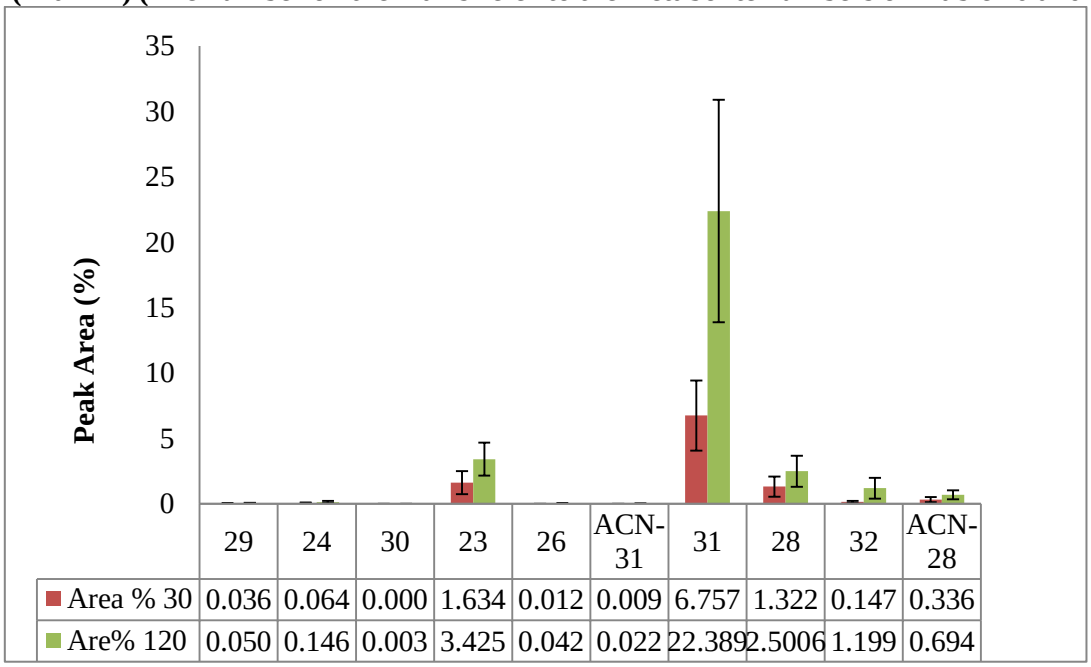
Figure3: Relative amounts of metabolites of (±)-mephedrone (4-MMC, 3a) formed after incubation with human hepatocytes (n = 4); ±SEM% area for 4-MMC (3a) = 95.8 ±1.3% (30 min) and 87.3 ±3.9% (120 min); U = unidentified metabolite (tR = 8.3 min; m/z = 194.1180) (The number on the x-axis refer to the metabolite numbers on Table 2 and Scheme 1).



	12	20	10	15	U	14	18	9	7	8	21	16	17	22
Area % 30	0	0	0	0	1	0.1	0	0.6	1.3	0	0.1	1	0	0
Area%120	0	0	0	0	2.9	0.1	0.4	2.5	3.9	0.1	0.7	1.8	0.1	0

The histogram shown in Figure 4 indicates that the major metabolite of 4-TFMMC (3b) was 4-(trifluoromethyl) ephedrine (31, *m/z*=234.10) that significantly increased after two hours incubation period by approximately 22 %. Two other metabolites were formed in moderate amounts and these were 23 (*m/z*= 248.0891) and 28 (*m/z*=218.0787). Comparing the two donors, it was found that donor (HU4215) tended to produce more abundant metabolite levels than donor (HU4226), particularly hydroxylated metabolites 31 (*m/z*=234.1098) 23 (*m/z*= 248.0891) and the demethylated metabolite 28 (*m/z*=218.0787). The Phase II metabolites of 4-TFMMC (3b) included a very low level of glucuronidated metabolite 30 (*m/z*=410.1059) which was observed after two hours incubation period as well as an acetylated metabolite 29 (*m/z* = 260.0892) which was found at a very low levels after 30 and 120 minutes incubations.

Figure 4: Relative amounts of metabolites of (±)-4'-(trifluoromethyl) methcathinone (4-TFMMC, 3b) formed after incubation with human hepatocytes (n = 4); ±SEM% area for 4-TFMMC (3b) = 89.7 ±4.5% (30 min) and 69.5 ±11.9% (120 min) (The number on the x-axis refer to the metabolite numbers on Table 26 and Scheme 12).



	29	24	30	23	26	ACN-31	31	28	32	ACN-28
Area % 30	0.036	0.064	0.000	1.634	0.012	0.009	6.757	1.322	0.147	0.336
Are% 120	0.050	0.146	0.003	3.425	0.042	0.022	22.389	2.5006	1.199	0.694

Aliquots were taken from the incubation to measure the half-life of 4-MMC (3a) and 4-TFMMC (3b) at 0, 30, 60, 90, and 120 minutes. The linear plots gave the slopes of 4-MMC (3a) and 4-TFMMC (3b) which were determined to be 1.0×10^{-3} and 3.2×10^{-3} respectively, and were used to estimate the metabolic rate constants (k_{met}).

In human hepatocytes the half –life of both drugs was higher than in rat cells and that might be expected due to the difference between the species metabolism rates. Figure 76 shows that 4-TFMMC (3b) was found to have a shorter half-life than 4-MMC (3a); 693.0 minutes and 216.6 minutes for 4-MMC (3a) and 4-TFMMC (3b), respectively. Surprisingly in human hepatocytes 4-TFMMC (3b) has a shorter half-life than 4-MMC (3a) but this might be attributed to the effects of the CF_3, which is para to the ketone group, activating the keto group so that it is more susceptible to reduction, and increasing the compounds lipophilicity and enhance its rate of metabolism by hepatic enzymes [19].

4. CONCLUSION:

The metabolism profiles of mephedrone (3a, 4-MMC) and its analogue, 4-TFMMC (3b), were analysed using human hepatocytes. Results indicated that the biotransformation rates for both compounds in human hepatocytes were lower than those observed in rat hepatocytes. This study also provided insights into the metabolic reactions of these substances in humans. Interestingly, although 4-TFMMC (3b) has a CF3 modification blocking metabolism at one site, it was metabolised faster than 4-MMC (3a). The reduced keto moiety in 3b formed dihydro-4-TFMMC (m/z = 234.1098), while the dominant pathway for 4-MMC (3a) involved demethylation (m/z = 164.1070) rather than dihydromephedrone (m/z = 180.1382). While Phase II metabolic pathways for both compounds were measurable in rat hepatocytes, human production was minimal, showing only trace amounts of some conjugates and significant variability between donors. This study demonstrates the comparative metabolic pathways between species by quantifying metabolite formation.

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