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Cytochrome P450 dependent metabolism of flumidione *in vitro* in rat and human microsomes and *in vivo* in rat.

Patrick M. Dansette¹, Assia Hessani¹, Virginie Siguret^{2,3}, Catherine Boisson-Vidal², Jean-Luc Boucher¹ and Blandine Dizier² with the participation of master students : Carole Berndt, Caroline Moreau, Idir Hamdidouche, Slimane Mokrani, Antoine Bienne, Shannon Pecnard and Victoria Rutman.

¹LCBPT, CNRS UMR 8601, Université Paris Descartes, Sorbonne Paris-Cité, PARIS, France

²INSERM UMR-S-1140 "Innovations thérapeutiques en hémostase" Faculté de Pharmacie, Université Paris Descartes, Sorbonne Paris-Cité, PARIS, France

³Service d'hématologie biologique (Pr A. Veyradier) - Hôpital Lariboisière, PARIS, France



INTRODUCTION : Flumidione (2-(4'-fluoro-phenyl)-phenylindane-1,3-dione) is an anti-vitamin K (AVK) marketed in 1967 in France and in Luxembourg (1-3). It is still the leading AVK in France in 2016 (around 800.000 patients, 82% of the market). However very little is known on the metabolism of this drug. In order to better understand the pharmacokinetics of this drug (4,5), we started to study the *in vitro* and *in vivo* metabolism of flumidione. The results below show a series of new metabolites both *in vitro* and *in vivo*.

METHODS: *In vivo* : Sprague Dawley rats weighting 200- 400 g were injected IP with flumidione (5 mg/mL solution in sodium bicarbonate) at 5 or 20 mg/kg. Blood was obtained at the tail at selected time and urine was collected.

In Vitro : Rat or human liver microsomes or recombinant Cytochrome P450 (CYP) (Supersomes Genteset) were incubated with flumidione (100 and 200 μM) for 10 to 60 min in presence of NADPH generating system. Reactions were stopped with one volume of CH₃CN/AcOH 9:1, centrifuged at 13000 g and supernatants were analyzed by HPLC/MS on a LCQ Advantage system or an Exactive Orbitrap (Thermo) using negative ESI. The metabolite M2 was obtained from semipreparative incubations, purified by HPLC and analyzed by NMR on a Bruker 500 MHz.

Synthesis: ¹³C₆ labeled flumidione was synthesized from ¹³C₆ labeled phthalic anhydride (6). 3-(4-Fluoro-phenyl)-4-hydroxy-coumarin (7), 3-(4-Fluorophenyl)-4-hydroxy-isocoumarin (8), 2-(4-hydroxyphenyl)-indane-1,3-dione (9) and 2-hydroxy-flumidione (10) were synthesised by modification of known methods.

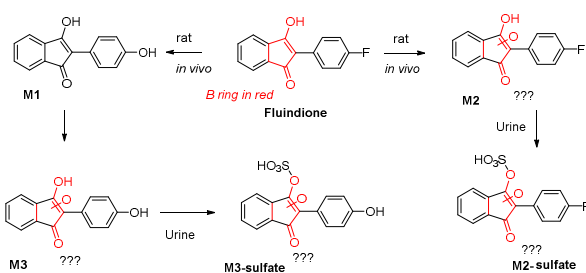
RESULTS :

***In vivo* :** Rats were treated by 5 and 20 mg/kg flumidione ¹³C₆/¹²C 1/1 mixture

Plasma : Analysis of plasma showed doubly labeled compounds with a doublet in the mass spectrum : flumidione F (m/z = 239/245, C₁₅H₉O₂) and metabolites: M1 (m/z = 237.0558/243.0760) corresponding to defluorination and hydroxylation, and identified by comparison with an authentic to 2-(4-hydroxyphenyl)-indane-1,3-dione and M2 (m/z = 255.0467/ 261.0668) corresponding to C₁₅H₈O₃F thus addition of an oxygen to flumidione. M2 was less polar than flumidione and had a UV spectrum nearly superimposable with that of flumidione (see below). A minor metabolite M3 also less (m/z = 253.0499/259.0700) corresponding to C₁₅H₉O₄ thus flumidione defluorinated and dihydroxylated.

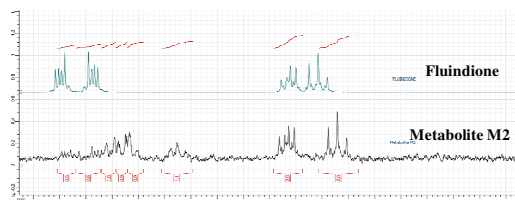
Urine : Analysis of urine showed the same metabolites in low amount plus series of more polar metabolites. They corresponded to sulfate esters: S2 was relatively abundant (m/z = 335.0006, C₁₅H₈O₆F₂) its MS and MS2 showed fragmentation at m/z = 255.0446 with loss of SO₃. S3 had a m/z = 333.0046 in MS and its MS2 spectrum showed fragmentation at m/z = 253.0499 with loss of SO₃. Thus we believe that S2 and S3 are the sulfate esters of M2 and M3.

Plasma and Human Urine: A poly-medicated volunteer took orally 10 mg of flumidione. Flumidione could be detected in plasma at 1, 2 and 6 hours. However the metabolites could only be found in very low amount (limit of detection).



Studies *In Vitro*:

Rat liver microsomes: Incubation of rat liver microsomes with 100 μM flumidione showed formation of a major metabolite identical to M2 with a m/z = 255.046 and a UV spectrum identical to that of flumidione. A semi-preparative incubation using 60 mL incubation medium allowed to isolate by HPLC about 0.3 mg of metabolite. Its proton NMR spectrum showed a disymmetrical molecule with all the ring A proton different. The fluorophenyl ring was not modified. We could not obtain a ¹³C spectrum for further identification. M2 was compared to synthetic 3-(4-fluoro-phenyl)-4-hydroxy-coumarin, 3-(4-fluoro-phenyl)-4-hydroxy-isocoumarin, 2-(4-hydroxy-phenyl)-indane-1,3-dione and 2-hydroxy-flumidione which were all different. Thus until now we don't have the structure of M2.



Human liver microsomes : Incubation with human liver microsomes showed that metabolite M2 was formed in presence of NADPH. Control without NADPH were blank. Its formation was inhibited by benzyl imidazole (50 μM) implicating cytochrome P450 catalysis. An impurity, 2-hydroxy-flumidione slowly increased on storage of incubations. It's a known oxidation product of flumidione by air.

Recombinant human P450: The formation of M2 was studied using insect cell microsomes expressing recombinant CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5. CYP1A2, 2B6, 2C9, 2D6, 3A4 and 3A5 could make this metabolite. CYP2C9 and CYP1A2 had best affinities (about 30 μM) as shown in Table 1.

Table 1 : Kinetic parameters for M2 formation by human P450 and liver microsomes

CYP or microsomes	1A2	2B6	2C9	2D6	3A4	Pool HLM
K _m (μM)	40	489	33	656	311	143
V _m (nmol/min/nmol P450)	2.2	3.7	3.2	5.9	8.9	0.3
V _m /K _m (L/min/nmol)	55	7.6	100	9	29	2

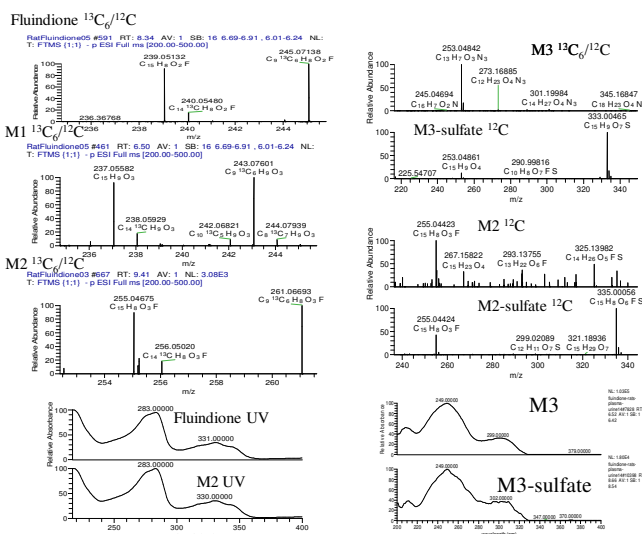
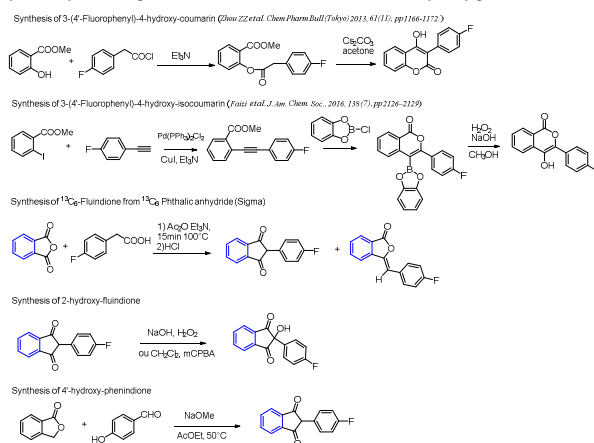


Figure 2 : Selected mass and UV spectra of flumidione and its metabolites

Synthesis of some potential metabolites and ¹³C₆ labeled flumidione:

In order to characterize M2, the chemical synthesis of several candidate molecules was performed: 2-hydroxy-flumidione, 2-(4'-fluorophenyl)-4-hydroxy-coumarin, 2-(4'-fluorophenyl)-4-hydroxy-isocoumarin, 4'-hydroxy-phenindione. Metabolite M2 did not correspond to any of the synthesized products. However M1 was identified as 4'-hydroxy-phenindione.



CONCLUSION :

- Flumidione is metabolized in rat and human microsome to oxidized metabolites corresponding to either an oxidation of the B ring of the indanedione (M2, m/z=255), or oxidation of the fluorophenyl with defluorination (M1, m/z = 237) and both reactions (M3, m/z = 253).
- These oxidations are P450 dependent and human recombinant CYP2C9 and CYP1A2 have good affinities for formation of M2 also formed by CYP3A4.
- *In vivo* in rat, the primary metabolites are found in plasma, but in urine one find the sulfate esters of M2 and M3.
- A tentative experiment in a human volunteer showed only flumidione and M2 in plasma and only traces of flumidione in urine.
- The metabolism of flumidione is mediated by several cytochrome P450 which explain why CYP2C9 is not a genetic determinant for its posology.
- Finally the structure of M2 and M3 remain to be characterized.

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