Supplements

List of supplementary figures and tables:

Table S1 – Specific tandem mass spectrometry settings applied for the detection and quantification of ivermectin and its metabolites.

Analyte	Q1 Mass [Da]	Q3 Mass [Da]	Dwell Time [ms]	DP [V]	EP [V]	CE [V]	CXP [V]
Ivermectin I	892.4	569.2	10	106	10	17	30
Ivermectin II	892.4	307.1	10	106	10	31	20
Ivermectin-d_2 I	894.4	571.3	10	131	10	17	30
Ivermectin-d_2 II	894.4	309.2	10	131	10	29	26
M1: 3''-O-desmethyl-H_2B_{1a}	878.0	307.1	10	106	10	31	20
M2: 4-hydroxy-H_2B_{1a}	908.0	307.1	10	106	10	31	20
M3: 26-hydroxy-H_2B_{1a}	908.0	323.0	10	106	10	31	20
M4: 3''-O-desmethyl, 4-hydroxy-H_2B_{1a}	894.0	307.0	10	106	10	31	20
M5: 4-hydroxy-H_2B_{1a} monosaccharide	764.0	307.0	10	106	10	31	20
M6: 3''-O-desmethyl, 26-hydroxy-H_2B_{1a}	894.0	323.0	10	106	10	31	20
M7: 26-hydroxy-H_2B_{1a} monosaccharide	764.0	323.0	10	106	10	31	20
M8: 4, 26-dihydroxy-H_2B_{1a}	924.0	323.0	10	106	10	31	20
M9: 24-hydroxy-H_2B_{1a}	908.1	323.0	10	106	10	31	20

H_2B_{1a}: ivermectin, mass filters (Q1 and Q3), Da: Daltons, ms: milliseconds, V: volts, DP: declustering potential, EP: entrance potential, CE: collision energy, CXP: Collision Cell Exit Potential.

Table S2 – Dual binary gradient flow program used for metabolism and fractioning assays.
Sample was loaded onto the analytical column using 2% mobile phase B. The initial total flow 1 and 3 was 0.1 mL/min (pump A and B) and 0.5 mL/min (pump C and D), respectively. Samples were diluted with mobile phase A within a T-union installed in front of the analytical column by pump C (total flow 3).

Time [min]	HPLC Module	Event	Parameter
0.50	Pumps	Pump B Conc.	2%
0.50	Pumps	Total Flow 1	0.6 mL/min
0.50	Pumps	Total Flow 3	0 mL/min
1.50	Pumps	Pump B Conc.	70%
2.00	Oven	Right Valve	0
9.00	Pumps	Pump B Conc.	87%
9.01	Pumps	Pump B Conc.	95%
9.50	Oven	Right Valve	1
11.50	Pumps	Pump B Conc.	95%
11.51	Pumps	Pump B Conc.	2%
12.00	Controller	Stop	

Pump B Conc.: Concentration of mobile phase B delivered by pump A and B.
Total Flow 1: Flow of pump A and B.
Total Flow 3: Flow of pump C and D.
For metabolism assays, the HPLC flow was directed into the MS (right valve: position 0) between 2.0 – 9.5 min, otherwise directed into the solvent waste (right valve: position 1).

Table S3 – Dual binary gradient flow program used for pharmacokinetic assays.
Sample was loaded onto the analytical column using 2% mobile phase B. The initial total flow 1 and 3 was 0.1 mL/min (pump A and B) and 0.5 mL/min (pump C and D), respectively. Samples were online diluted with 2% mobile phase B (98% mobile phase A) within a T-union installed in front of the analytical column.

Time [min]	HPLC Module	Event	Parameter
0.50	Pumps	Pump B Conc.	2 %
0.50	Pumps	Total Flow 1	0.6 mL/min
0.50	Pumps	Total Flow 3	0 mL/min
1.00	Pumps	Pump B Conc.	60 %
2.50	Oven	Right Valve	0
5.00	Pumps	Pump B Conc.	82 %
5.25	Pumps	Pump B Conc.	95 %
5.50	Oven	Right Valve	1
6.00	Pumps	Pump B Conc.	95 %
6.01	Pumps	Pump B Conc.	2 %
6.50	Controller	Stop	

Pump B Conc.: Concentration of mobile phase B delivered by pump A and B.
Total Flow 1: Flow of pump A and B.
Total Flow 3: Flow of pump C and D.

The HPLC flow was directed into the MS (right valve: position 0) between 2.5 – 5.5 min, otherwise directed into the solvent waste (right valve: position 1).

Figure S1 – Chromatogram before/after fractioning of the ivermectin metabolites.

Ivermectin (H_2B_{1a}) metabolites: **M1**: 3''-O-desmethyl-H_2B_{1a}, **M2**: 4-hydroxy-H_2B_{1a}, **M3**: 26-hydroxy-H_2B_{1a}, **M4**: 3''-O-desmethyl, 4-hydroxy-H_2B_{1a}, **M5**: 4-hydroxy-H_2B_{1a} monosaccharide, **M6**: 3''-O-desmethyl, 26-hydroxy-H_2B_{1a}, **M7**: 26-hydroxy-H_2B_{1a} monosaccharide, **M8**: 4, 26-dihydroxy-H_2B_{1a}, **M9**: 24-hydroxy-H_2B_{1a}.

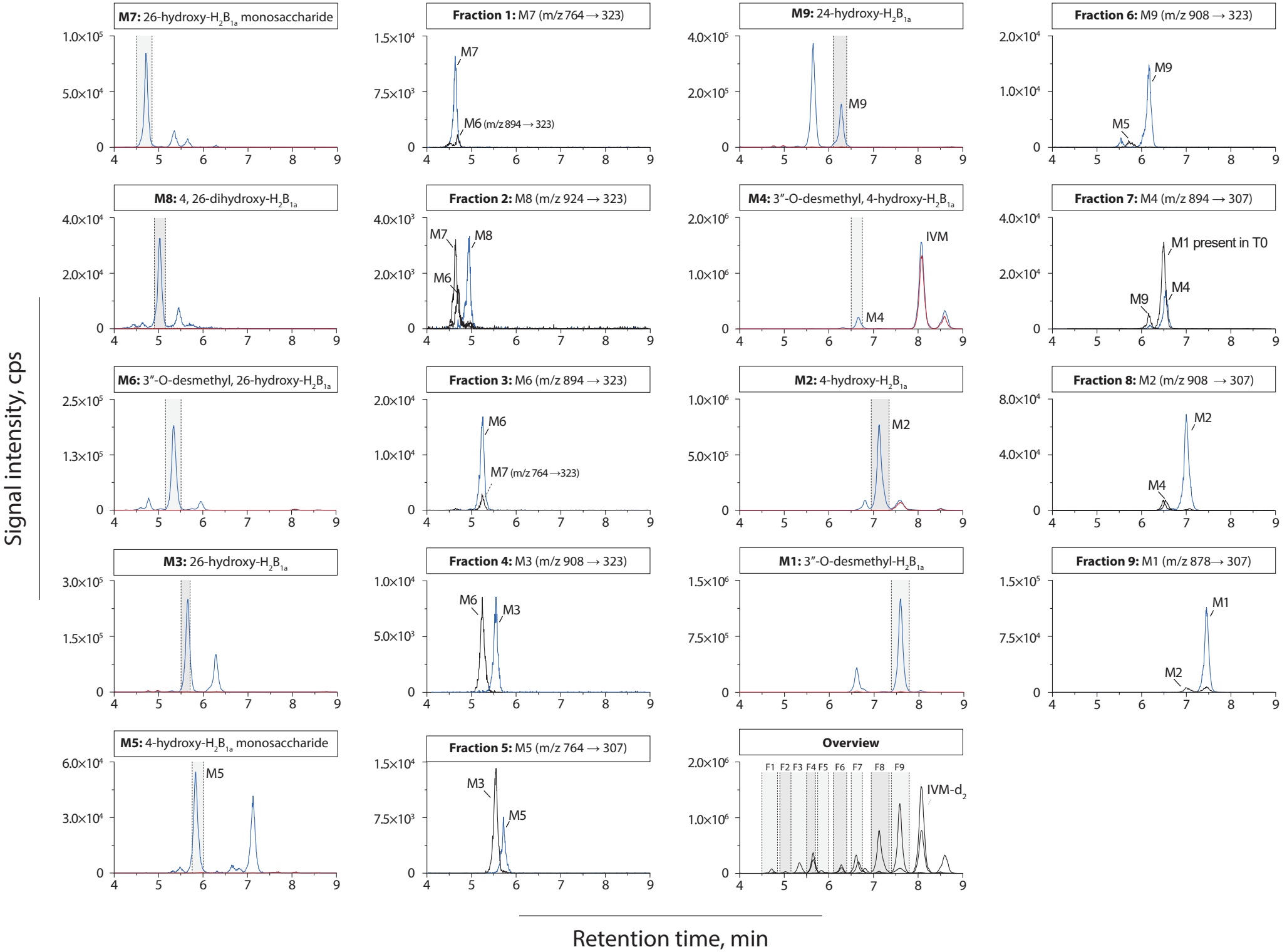

M7: 26-hydroxy-H2B1a monosaccharide
Fraction 1: M7 (m/z 764 → 323)
M9: 24-hydroxy-H2B1a
Fraction 6: M9 (m/z 908 → 323)
M8: 4, 26-dihydroxy-H2B1a
Fraction 2: M8 (m/z 924 → 323)
M4: 3″-O-desmethyl, 4-hydroxy-H2B1a
Fraction 7: M4 (m/z 894 → 307)
M6: 3″-O-desmethyl, 26-hydroxy-H2B1a
Fraction 3: M6 (m/z 894 → 323)
M2: 4-hydroxy-H2B1a
Fraction 8: M2 (m/z 908 → 307)
M3: 26-hydroxy-H2B1a
Fraction 4: M3 (m/z 908 → 323)
M1: 3″-O-desmethyl-H2B1a
Fraction 9: M1 (m/z 878→ 307)
M5: 4-hydroxy-H2B1a monosaccharide
Fraction 5: M5 (m/z 764 → 307)
Overview
M7
M6 (m/z 894 → 323)
M8
M6
IVM
M4
M1 present in T0
M9
M4
M6
M7 (m/z 764 →323)
M2
M4
M6
M3
M2
M1
M3
M5
F1 F2 F3 F4 F5 F6 F7 F8 F9
IVM-d2
Signal intensity, cps
Retention time, min

Table S4 – Ivermectin metabolite composition (M1-M9) of each fraction (F1-F9).

Numbers indicate the mean peak area (n=4) in percentage to the largest observed signal of the same metabolite. The standard deviation is given in parentheses. Values under 10% are not included. Bold numbers indicate which metabolite was considered for each fraction (e.g. 100% of M9 in fraction 6 [F6]).

	M7, %	**M8, %**	**M6, %**	**M3, %**	**M5, %**	**M9, %**	**M4, %**	**M2, %**	**M1, %**
F1: 4.50 − 4.85 min	**100**								
F2: 4.90 − 5.15 min	24.4 (5.4)	**100**							
F3: 5.15 − 5.50 min		21.1 (4.4)	**100**						
F4: 5.50 − 5.70 min			40.4 (6.5)	**66 (5.3)**					
F5: 5.75 − 6.00 min				100	**100**				
F6: 6.10 − 6.40 min					29.3 (5.7)	**100**			
F7: 6.50 − 6.75 min						28.9 (2.7)	**100**		
F8: 6.95 − 7.35 min					10.2 (2.2)		70.3 (9.9)	**100**	
F9: 7.40 − 7.80 min								13.3 (1.3)	**100**

Ivermectin (H_2B_{1a}) metabolites: **M1**: 3''-O-desmethyl-H_2B_{1a}, **M2**: 4-hydroxy-H_2B_{1a}, **M3**: 26-hydroxy-H_2B_{1a}, **M4**: 3''-O-desmethyl, 4-hydroxy-H_2B_{1a}, **M5**: 4-hydroxy-H_2B_{1a} monosaccharide, **M6**: 3''-O-desmethyl, 26-hydroxy-H_2B_{1a}, **M7**: 26-hydroxy-H_2B_{1a} monosaccharide, **M8**: 4, 26-dihydroxy-H_2B_{1a}, **M9**: 24-hydroxy-H_2B_{1a}.

Table S5 – Extended pharmacokinetic parameters of ivermectin metabolites in whole blood for 12 subjects after a dose of 12 mg ivermectin.

Blood was collected on volunteers pre- and up to 72h post-ivermectin dose (Duthaler et al., 2019). Statistics on Non Compartmental Analysis results for the pharmacokinetic parameters. Pharmacokinetic parameters are showed as mean (± SE). The ratio Area/IS Area is used for the chromatographic peak area of metabolites, whereas for ivermectin quantitative data with SD was taken from Duthaler et al., 2019.
The least abundant metabolites M5, M7, M8 and M9 were excluded from the NCA analysis.

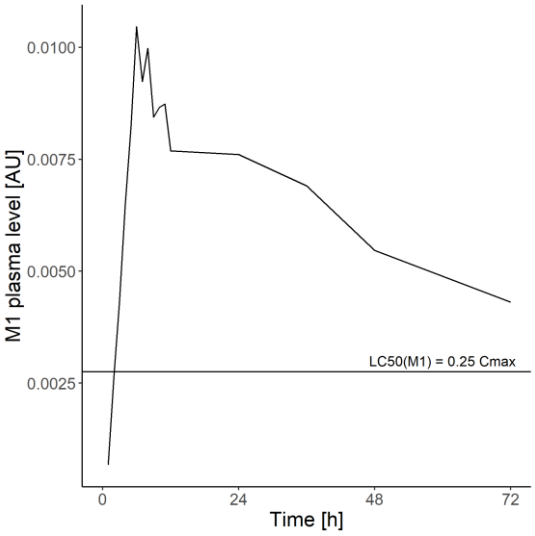
IVM metabolite	T_{max} [h]	C_{max} [ratio]	Half-life [h]	AUC [ratio/h]	MRT_{last} [h]
M1	7.0 ± 0.5	0.011 ± 0.001	54.2 ± 4.7	0.48 ± 0.06	33.8 ± 0.9
M2	6.3 ± 0.3	0.010 ± 0.0010	31.9 ± 3.4	0.24 ± 0.03	25.8 ± 0.7
M3	5.3 ± 0.2	0.003 ± 0.0003	15.9 ± 2.8	0.04 ± 0.005	13.9 ± 1.3
M4	6.0 ± 0.2	0.004 ± 0.0004	57.5 ± 13.2	0.09 ± 0.01	29.4 ± 1.7
M5	6.4 ± 0.4	0.006 ± 0.0001	27.1 ± 9.4	0.004 ± 0.0006	8.3 ± 0.8
M6	5.4 ± 0.4	0.002 ± 0.0002	32.4 ± 4.1	0.02 ± 0.003	18.3 ± 1.6
M7	6.1 ± 0.4	0.001 ± 0.0001	32.5 ± 8.3	0.01 ± 0.002	13.9 ± 2.3
M8	5.0 ± 0.3	0.0004 ± 0.00004	04.1 ± 0.9	0.001 ± 0.002	5.0 ± 0.4
M9	6.0 ± 0.8	N/A	N/A	N/A	N/A
parent	**T_{max} [h]**	**C_{max} [ng/mL]**	**Half-life [h]**	**AUC [ng/mL.h]**	
IVM	4.4 ± 1.4	70.7 ± 16.1	38.9 ± 20.8	1743.5 ± 602.5	

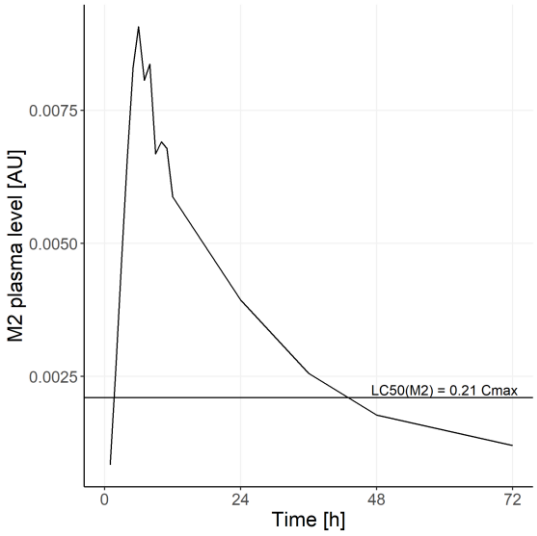
IVM metabolite	CL/F [L/h]	Vz/F [L]	Time above LC_{50} [h]
M1	19.9 ± 4.7	1389.1 ± 228.8	69.0
M2	48.1 ± 7.8	2058.1 ± 278.8	34.0
M3	305.9 ± 64.8	5306.1 ± 400.5	
M4	91.6 ± 16.9	5927.2 ± 618.1	
M5	1268.2 ± 261.8	27443.6 ± 3734.5	
M6	419.6 ± 67.2	17025.5 ± 1915.5	
M7	814.5 ± 175.5	21581.2 ± 3357.1	
M8	4528.5 ± 646.4	25906.2 ± 1993.1	
M9	N/A	N/A	
parent	**CL/F [L/h]**	**Vz/F [L]**	
IVM	7.7 ± 2.8	382.6 ± 106.1	69.3

Ivermectin (H_2B_{1a}) metabolites: **M1**: 3''-O-desmethyl-H_2B_{1a}, **M2**: 4-hydroxy-H_2B_{1a}, **M3**: 26-hydroxy-H_2B_{1a}, **M4**: 3''-O-desmethyl, 4-hydroxy-H_2B_{1a}, **M5**: 4-hydroxy-H_2B_{1a} monosaccharide, **M6**: 3''-O-desmethyl, 26-hydroxy-H_2B_{1a}, **M7**: 26-hydroxy-H_2B_{1a} monosaccharide, **M8**: 4, 26-dihydroxy-H_2B_{1a}, **M9**: 24-hydroxy-H_2B_{1a}.

Standard error (SE); time of peak concentration (T_{max}); peak concentration (C_{max}); area under the curve from the time of dosing to the last measurable concentration (AUC); Mean residence time from the time of dosing to the time of the last measurable concentration (MRT_{last}); clearance (CL) over bioavailability (F) based on concentration at the final observation time (CL/F); volume of distribution over bioavailability (V/F).

Drug level above LC_{50} in whole blood for ivermectin (**IVM**), 3''-O-desmethyl-H_2B_{1a} (**M1**) and 4-hydroxy-H_2B_{1a} (**M2**).





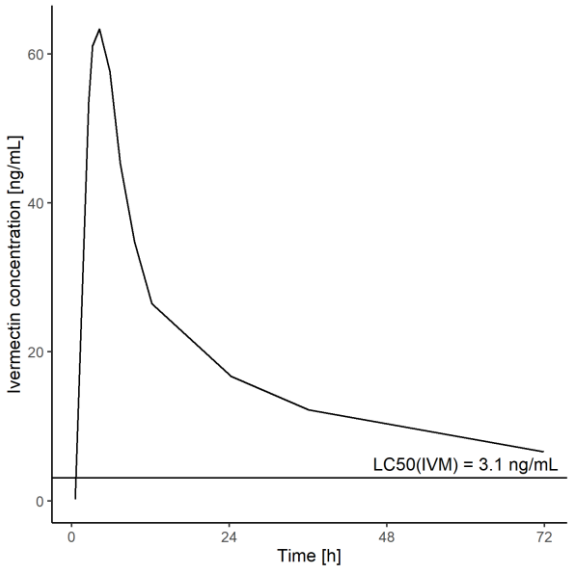
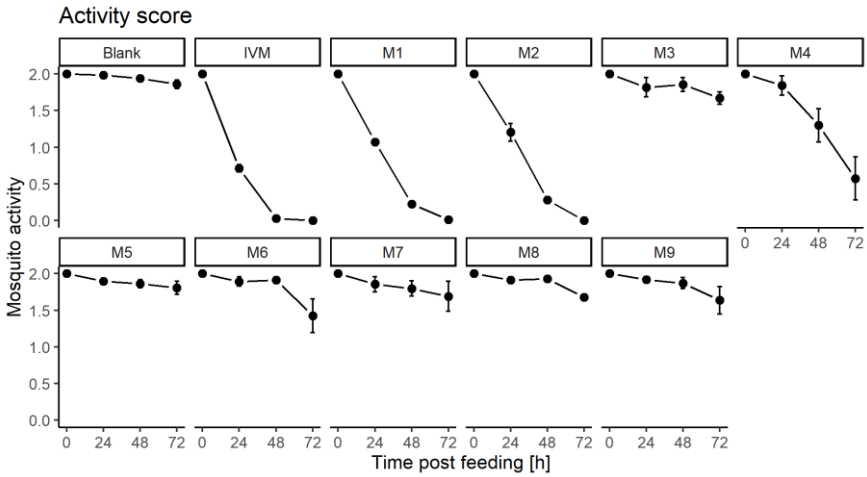


Figure S2 – Screening for ivermectin metabolites effect on mosquito activity.

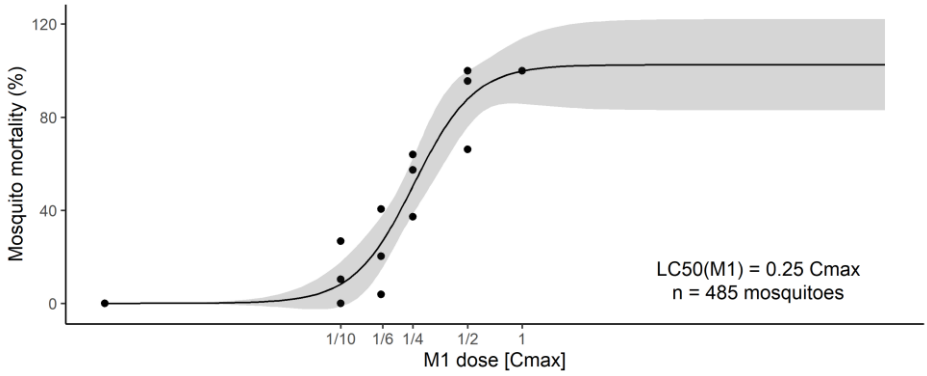
Anopheles stephensi mosquitoes were treated with blank human blood (**blank**), or blood containing either ivermectin (**IVM**, 50 ng/mL), or an ivermectin metabolite (M1-M9): **M1**: 3''-O-desmethyl-H_2B_{1a}, **M2**: 4-hydroxy-H_2B_{1a}, **M3**: 26-hydroxy-H_2B_{1a}, **M4**: 3''-O-desmethyl, 4-hydroxy-H_2B_{1a}, **M5**: 4-hydroxy-H_2B_{1a} monosaccharide, **M6**: 3''-O-desmethyl, 26-hydroxy-H_2B_{1a}, **M7**: 26-hydroxy-H_2B_{1a} monosaccharide, **M8**: 4, 26-dihydroxy-H_2B_{1a}, **M9**: 24-hydroxy-H_2B_{1a}.
Three batches of mosquitoes, with an average of 26 mosquitoes/condition, were used per treatment group. Mean mosquito activity was assessed after 24h, 48h and 72h. Error bars correspond to the range. Mosquito's activity score: +2: The mosquito can fly and reach the top of the cup, it stays mainly on the top of the cup; +1: The mosquito is staying on the bottom of the cup, it is not able to fly but maybe can cover short distances on the ground; 0: No movements observed upon physical contact, the mosquito is classified as dead.

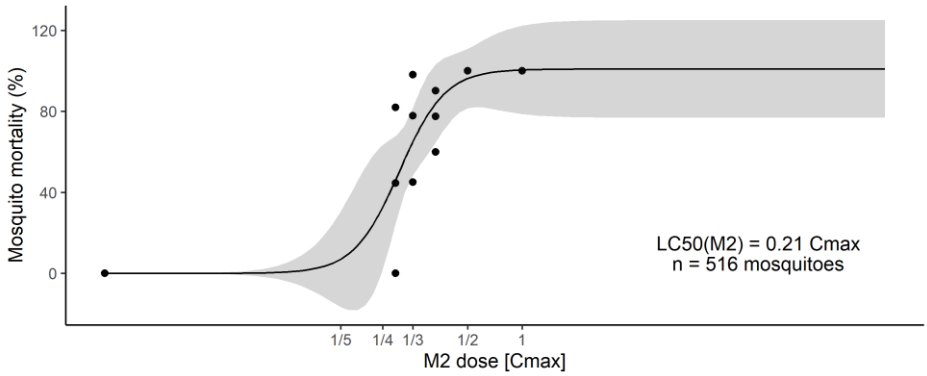


IVM metabolite	Time post feeding [h]	Average activity score	SE	IVM metabolite	Time post feeding [h]	Average activity score	SE
IVM	0	2.00	0.00	**M5**	0	2.00	0.00
IVM	24	0.71	0.05	**M5**	24	1.90	0.01
IVM	48	0.03	0.02	**M5**	48	1.86	0.06
IVM	72	0	0.00	**M5**	72	1.81	0.09
M1	0	2.00	0.00	**M6**	0	2.00	0.00
M1	24	1.07	0.04	**M6**	24	1.89	0.07
M1	48	0.23	0.02	**M6**	48	1.91	0.01
M1	72	0.01	0.01	**M6**	72	1.42	0.23
M2	0	2.00	0.00	**M7**	0	2.00	0.00
M2	24	1.20	0.12	**M7**	24	1.85	0.11
M2	48	0.28	0.05	**M7**	48	1.80	0.10
M2	72	0	0.00	**M7**	72	1.69	0.21
M3	0	2.00	0.00	**M8**	0	2.00	0.00
M3	24	1.82	0.13	**M8**	24	1.91	0.01
M3	48	1.86	0.09	**M8**	48	1.93	0.02
M3	72	1.67	0.08	**M8**	72	1.68	0.04
M4	0	2.00	0.00	**M9**	0	2.00	0.00
M4	24	1.84	0.13	**M9**	24	1.92	0.03
M4	48	1.30	0.23	**M9**	48	1.87	0.08
M4	72	0.57	0.29	**M9**	72	1.64	0.19
blank	0	2.00	0.00				
blank	24	1.98	0.02				
blank	48	1.94	0.04				
blank	72	1.86	0.06				

Figure S3 – Fit of the non-linear model to calculate IVM, M1 and M2 LC_{50}.

Multiple doses/concentrations of drug (**IVM:** ivermectin, **M1**: 3''-O-desmethyl-H_2B_{1a}, **M2**: 4-hydroxy-H_2B_{1a}) were orally fed to *Anopheles stephensi* mosquitoes to determine the lethal concentration that killed 50% of the mosquitoes (LC_{50}) 72h after treatment. The experiment was performed in triplicate, with an average of 24 [8 – 54] mosquitoes per drug dilution and a total number of mosquito fed per drug (n). A plot of the observed mortality versus the dose/concentration fed to the mosquito (black dots) was overlaid with the plot of the confidence bands (grey) around the fitted regression curve (black line).





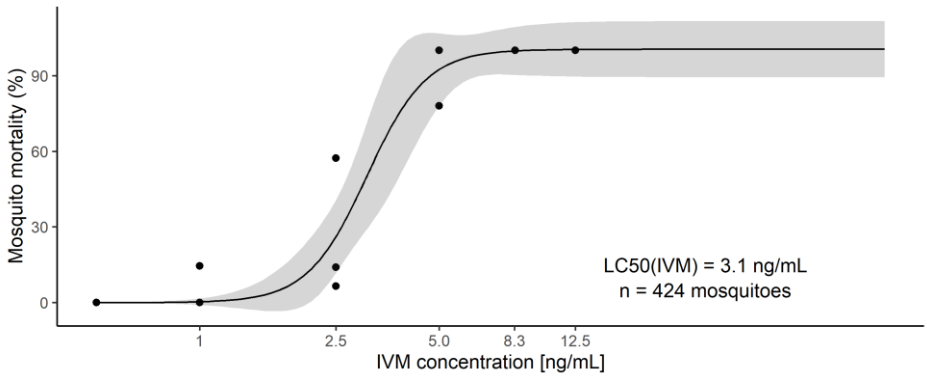
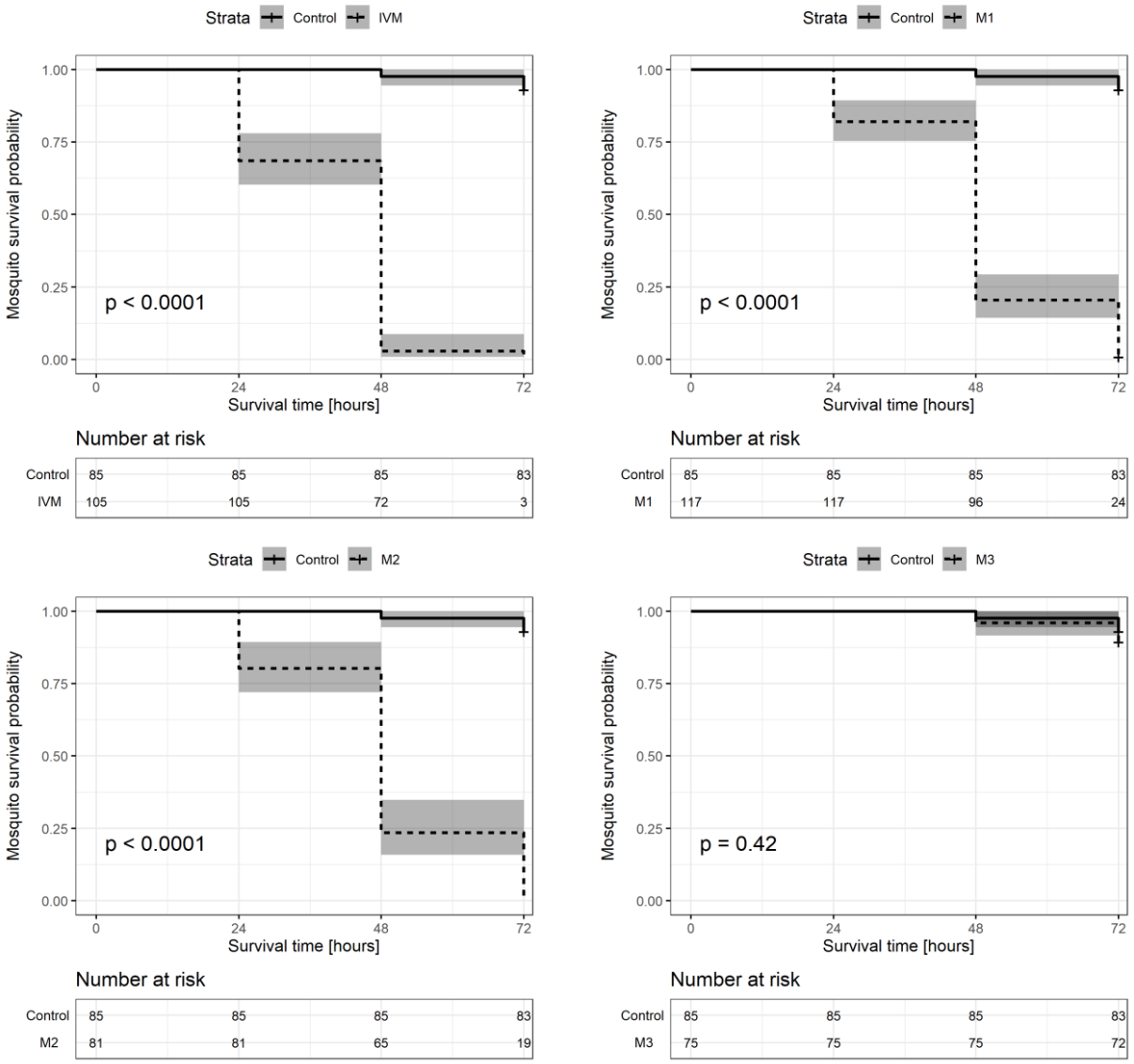
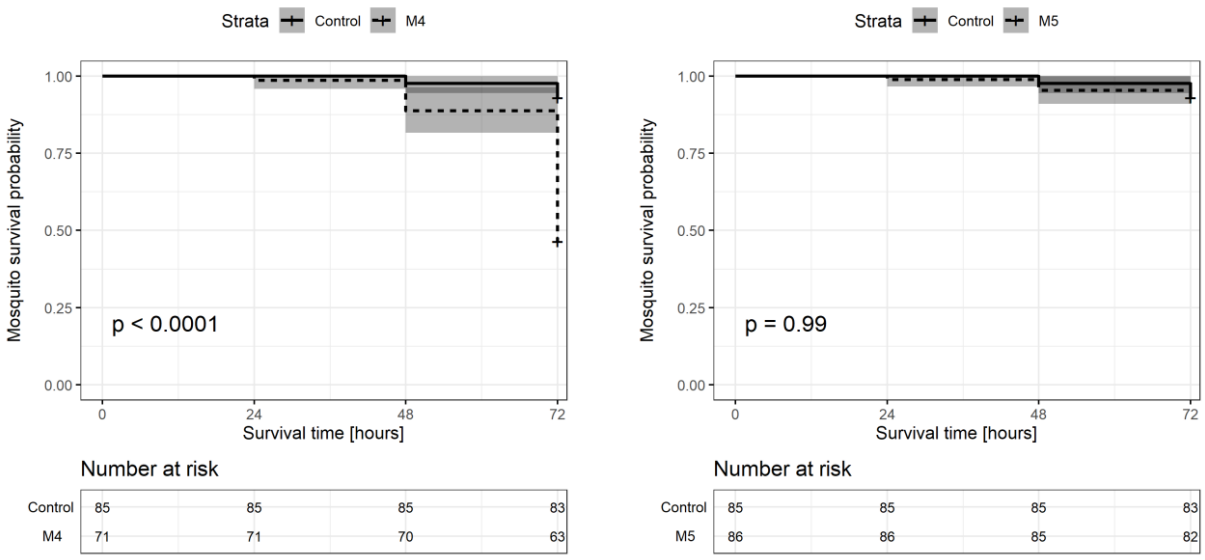


Figure S4 – *Anopheles stephensi* survival probability after absorbing blood meals containing ivermectin and ivermectin metabolites.

Anopheles stephensi mosquitoes were given a blood meal that contained ivermectin (IVM) or metabolites (M1–M9) concentrations corresponding to those interpolated from the IVM pharmacokinetic curve, and then monitored for survival over time (at 24h, 48h and 72h post feeding). Mosquitoes that exhibited significantly reduced survival (Log rank test) compared to control (blood meal contained only PBS) have a p-value < 0.0001.





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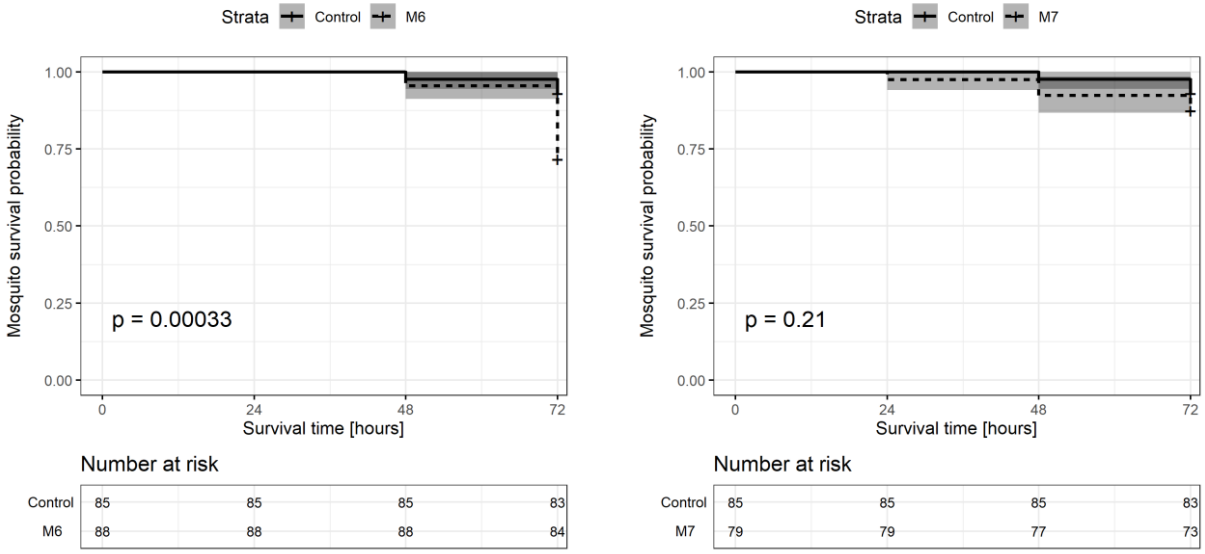

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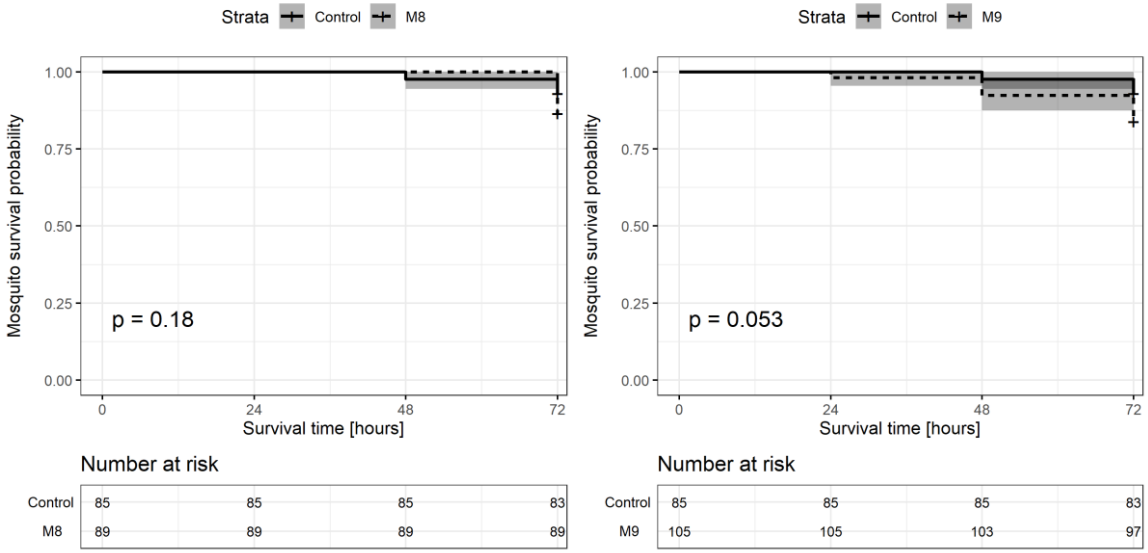

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128 Ivermectin (H_2B_{1a}) metabolites: **M1**: 3''-O-desmethyl-H_2B_{1a}, **M2**: 4-hydroxy-H_2B_{1a}, **M3**: 26-hydroxy-H_2B_{1a}, **M4**: 3''-
129 O-desmethyl, 4-hydroxy-H_2B_{1a}, **M5**: 4-hydroxy-H_2B_{1a} monosaccharide, **M6**: 3''-O-desmethyl, 26-hydroxy-H_2B_{1a}, **M7**:
130 26-hydroxy-H_2B_{1a} monosaccharide, **M8**: 4, 26-dihydroxy-H_2B_{1a}, **M9**: 24-hydroxy-H_2B_{1a}.