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: desmethyl-H_2B_{1a}	878.0	307.1	10	106	10	31	20
M2: hydroxy-H_2B_{1a}	908.0	307.1	10	106	10	31	20
M3: hydroxy-H_2B_{1a}	908.0	323.0	10	106	10	31	20
M4: desmethyl, hydroxy-H_2B_{1a}	894.0	307.0	10	106	10	31	20
M5: hydroxy-H_2B_{1a} monosaccharide	764.0	307.0	10	106	10	31	20
M6: desmethyl, hydroxy-H_2B_{1a}	894.0	323.0	10	106	10	31	20
M7: hydroxy-H_2B_{1a} monosaccharide	764.0	323.0	10	106	10	31	20
M8: dihydroxy-H_2B_{1a}	924.0	323.0	10	106	10	31	20
M9: 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.
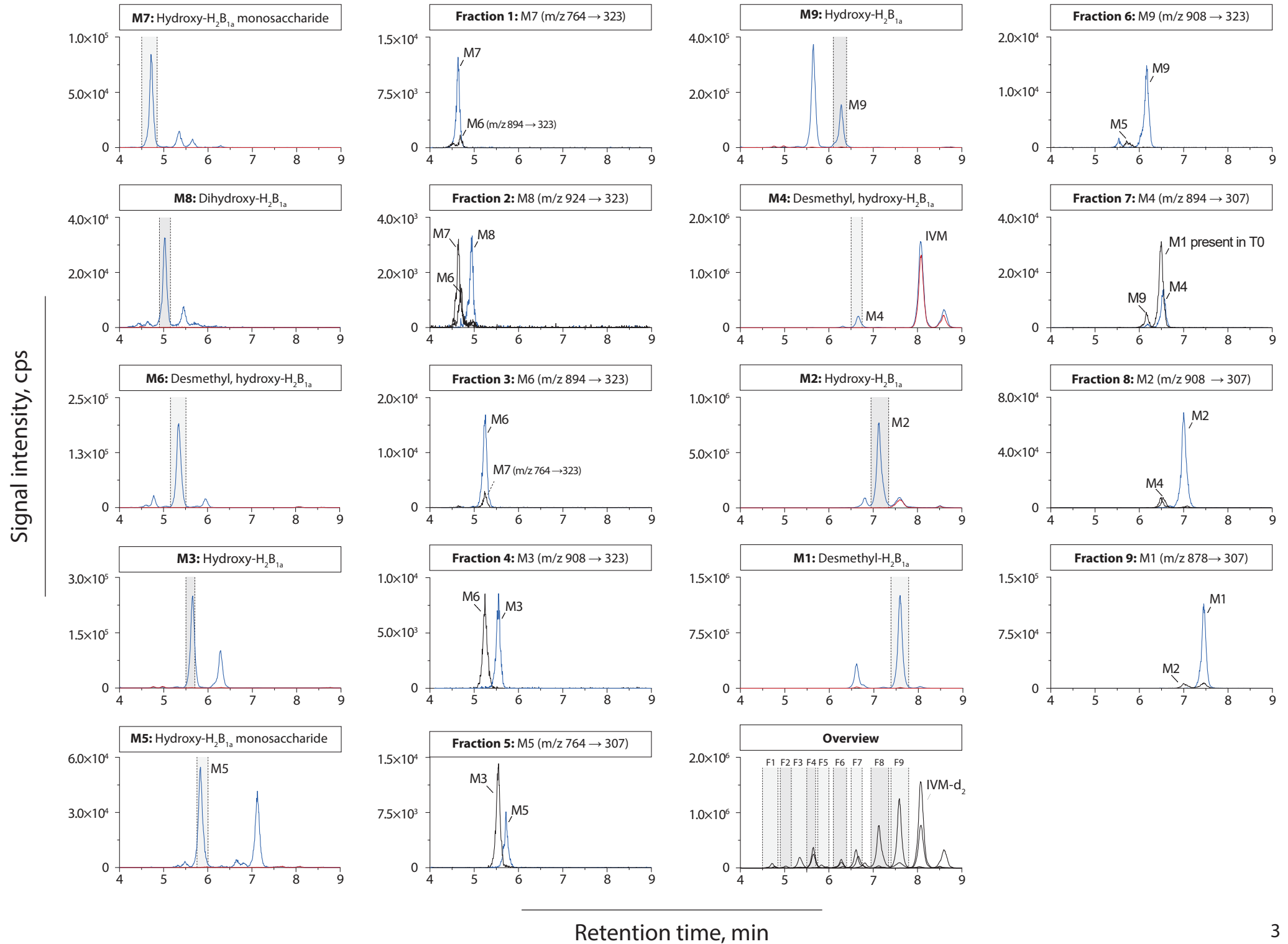
Table S4 – Ivermectin metabolite composition (M1-M9) of each fraction (F1-F9).
Numbers indicate the mean peak area (n=4) in percentage to the largest observed signal of the same metabolite. The standard deviation is given in parentheses. Values under 10% are not included. Bold numbers indicate which 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**: Desmethyl-H_2B_{1a}, **M2**: Hydroxy-H_2B_{1a}, **M3**: Hydroxy-H_2B_{1a}, **M4**: Desmethyl, hydroxy-H_2B_{1a}, **M5**: Hydroxy-H_2B_{1a} monosaccharide, **M6**: Desmethyl, hydroxy-H_2B_{1a}, **M7**: Hydroxy-H_2B_{1a} monosaccharide, **M8**: Dihydroxy-H_2B_{1a}, **M9**: Hydroxy-H_2B_{1a}.

Table S5a-c – Comparison of ivermectin metabolite C$_{max}$ pharmacokinetic levels with spiked blank blood samples.

Maximal analyte peak area of metabolite 1-9 (M1-M9) measured for PK subject 1-12 in 50 µL blood. An aliquot of 50 µL blank blood of PK subject 2, 4, and 6 was spiked with 10 µL of metabolite fraction (F1: M7, F2: M8, F3: M6, F4: M3, F5: M5, F6: M9, F7: M4, F8: M2, F9: M1). The mean peak area of the spiked blood samples was compared with the mean area of the C$_{max}$ samples to calculate the volume of metabolite fraction required to obtain equal values.

Table S5a – Data of ivermectin metabolites M1-M3

PK Subject ID	Observed C$_{max}$ of M1	M1 spiked to blank blood	Observed C$_{max}$ of M2	M2 spiked to blank blood	Observed C$_{max}$ of M3	M3 spiked to blank blood
	Peak area (counts)	Peak area (counts)	Peak area (counts)	Peak area (counts)	Peak area (counts)	Peak area (counts)
1	1.95E+04		1.84E+04		6.21E+03	
2	1.01E+04	5.07E+04	9.36E+03	3.57E+04	3.33E+03	2.97E+03
3	1.39E+04		1.31E+04		4.98E+03	
4	8.48E+03	6.10E+04	7.42E+03	4.78E+04	2.70E+03	3.46E+03
5	9.52E+03		9.63E+03		3.63E+03	
6	2.33E+04	7.58E+04	2.00E+04	3.11E+04	5.52E+03	3.35E+03
7	1.56E+04		1.39E+04		4.44E+03	
8	1.79E+04		1.25E+04		4.04E+03	
9	1.28E+04		1.08E+04		3.41E+03	
10	1.60E+04		1.50E+04		4.42E+03	
11	2.28E+04		1.64E+04		4.22E+03	
12	1.78E+04		1.59E+04		4.51E+03	
Mean	1.56E+04	6.25E+04	1.35E+04	3.82E+04	4.28E+03	3.26E+03
Difference spiked blood / C$_{max}$		4.0		2.8		0.8
Vol. of fraction required for 50 µL blood		2.5		3.5		13.1
Vol. of fraction required for 3 mL blood		**150**		**212**		**788**

Ivermectin (H$_2$B$_{1a}$) metabolites: **M1**: Desmethyl-H$_2$B$_{1a}$, **M2**: Hydroxy-H$_2$B$_{1a}$, **M3**: Hydroxy-H$_2$B$_{1a}$, **M4**: Desmethyl, hydroxy-H$_2$B$_{1a}$, **M5**: Hydroxy-H$_2$B$_{1a}$ monosaccharide, **M6**: Desmethyl, hydroxy-H$_2$B$_{1a}$, **M7**: Hydroxy-H$_2$B$_{1a}$ monosaccharide, **M8**: Dihydroxy-H$_2$B$_{1a}$, **M9**: Hydroxy-H$_2$B$_{1a}$.

Table S5b – Data of ivermectin metabolites M4-M6.

PK Subject ID	Observed C_{max} of M4	M4 spiked to blank blood	Observed C_{max} of M5	M5 spiked to blank blood	Observed C_{max} of M6	M6 spiked to blank blood
	Peak area (counts)	Peak area (counts)	Peak area (counts)	Peak area (counts)	Peak area (counts)	Peak area (counts)
1	6.13E+03		7.86E+02		3.04E+03	
2	4.65E+03	8.84E+03	5.85E+02	2.73E+03	1.56E+03	1.03E+04
3	5.03E+03		1.01E+03		2.10E+03	
4	4.08E+03	1.01E+04	7.22E+02	3.40E+03	1.60E+03	6.99E+03
5	2.43E+03		6.06E+02		1.15E+03	
6	7.98E+03	5.39E+03	1.25E+03	3.27E+03	3.23E+03	7.74E+03
7	4.09E+03		8.58E+02		1.62E+03	
8	5.21E+03		6.84E+02		1.82E+03	
9	4.12E+03		6.59E+02		1.80E+03	
10	5.38E+03		7.23E+02		1.99E+03	
11	5.67E+03		8.95E+02		2.12E+03	
12	5.92E+03		7.29E+02		2.69E+03	
Mean	5.06E+03	8.11E+03	7.92E+02	3.13E+03	2.06E+03	8.33E+03
Difference spiked blood / C_{max}		1.6		4.0		4.0
Vol. of fraction required for 50 µL blood		6.2		2.5		2.5
Vol. of fraction required for 3 mL blood		**374***		**152**		**148**

Ivermectin (H_2B_{1a}) metabolites: **M4**: Desmethyl, hydroxy-H_2B_{1a}, **M5**: Hydroxy-H_2B_{1a} monosaccharide, **M6**: Desmethyl, hydroxy-H_2B_{1a},

Table S5c – Data of ivermectin metabolites M7-M9.

PK Subject ID	Observed C_{max} of M7	M7 spiked to blank blood	Observed C_{max} of M8	M8 spiked to blank blood	Observed C_{max} of M9	M9 spiked to blank blood
	Peak area (counts)	Peak area (counts)	Peak area (counts)	Peak area (counts)	Peak area (counts)	Peak area (counts)
1	1.31E+03		7.02E+02		N/A	
2	7.70E+02	5.49E+03	5.34E+02	2.05E+03	N/A	1.09E+04
3	9.70E+02		4.91E+02		N/A	
4	7.68E+02	6.70E+03	3.41E+02	1.37E+03	N/A	1.24E+04
5	8.12E+02		3.43E+02		N/A	
6	1.39E+03	4.86E+03	6.41E+02	1.54E+03	N/A	1.08E+04
7	1.26E+03		3.42E+02		N/A	
8	1.09E+03		4.91E+02		N/A	
9	7.88E+02		4.29E+02		N/A	
10	9.63E+02		4.93E+02		N/A	
11	1.21E+03		2.79E+02		N/A	
12	9.57E+02		4.55E+02		N/A	
Mean	1.02E+03	5.68E+03	4.62E+02	1.65E+03	N/A	1.14E+04
Difference spiked blood / C_{max}		5.5		3.6		N/A
Vol. of fraction required for 50 µL blood		1.8		2.8		N/A
Vol. of fraction required for 3 mL blood		**108**		**168**		**120***

Ivermectin (H_2B_{1a}) metabolites: **M7**: Hydroxy-H_2B_{1a} monosaccharide, **M8**: Dihydroxy-H_2B_{1a}, **M9**: Hydroxy-H_2B_{1a}.

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**: Desmethyl-H_2B_{1a}, **M2**: Hydroxy-H_2B_{1a}, **M3**: Hydroxy-H_2B_{1a}, **M4**: Desmethyl, hydroxy-H_2B_{1a}, **M5**: Hydroxy-H_2B_{1a} monosaccharide, **M6**: Desmethyl, hydroxy-H_2B_{1a}, **M7**: Hydroxy-H_2B_{1a} monosaccharide, **M8**: Dihydroxy-H_2B_{1a}, **M9**: 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.
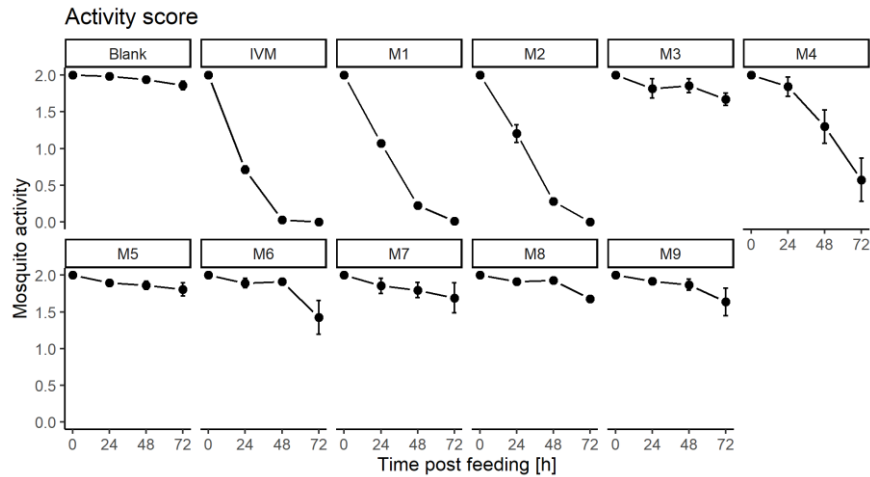

IVM metabolite	Time post feeding [h]	Average activity score	SE	IVM metabolite	Time post feeding [h]	Average activity score	SE
IVM	0	2.00	0.00	M5	0	2.00	0.00
IVM	24	0.71	0.05	M5	24	1.90	0.01
IVM	48	0.03	0.02	M5	48	1.86	0.06
IVM	72	0	0.00	M5	72	1.81	0.09
M1	0	2.00	0.00	M6	0	2.00	0.00
M1	24	1.07	0.04	M6	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**: Desmethyl-H$_2$B$_{1a}$, **M2**: Hydroxy-H$_2$B$_{1a}$) were orally fed to *Anopheles stephensi* mosquitoes to determine the lethal concentration that killed 50% of the mosquitoes (LC$_{50}$) 72h after treatment. The blood samples of the participants of the pharmacokinetics trial (Duthaler et al., 2019) were analyzed to derive the maximal blood level (C$_{max}$) for ivermectin, M1 and M2 **(Figure 2)**. An administration of 12 mg ivermectin yielded a C$_{max}$ of 50 ng/mL for ivermectin, therefore LC$_{50}$ was evaluated using ivermectin concentrations ranging between 1–12.5 ng/mL. For the metabolites M1 and M2, C$_{max}$ corresponds to the maximal intensity (peak area, counts) from the metabolite peak intensity time plots. *Anopheles stephensi* were treated with different dilutions of ivermectin ($^1/_4$ C$_{max}$, $^1/_6$ C$_{max}$, $^1/_{10}$ C$_{max}$, $^1/_{20}$ C$_{max}$, $^1/_{50}$ C$_{max}$), M1 (C$_{max}$, $^1/_2$ C$_{max}$, $^1/_4$ C$_{max}$, $^1/_6$ C$_{max}$, $^1/_{10}$ C$_{max}$) and M2 (C$_{max}$, $^1/_2$ C$_{max}$, $^1/_3$ C$_{max}$, $^1/_4$ C$_{max}$, $^1/_5$ C$_{max}$). 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). LC$_{50}$ summarized in **Table 2**.
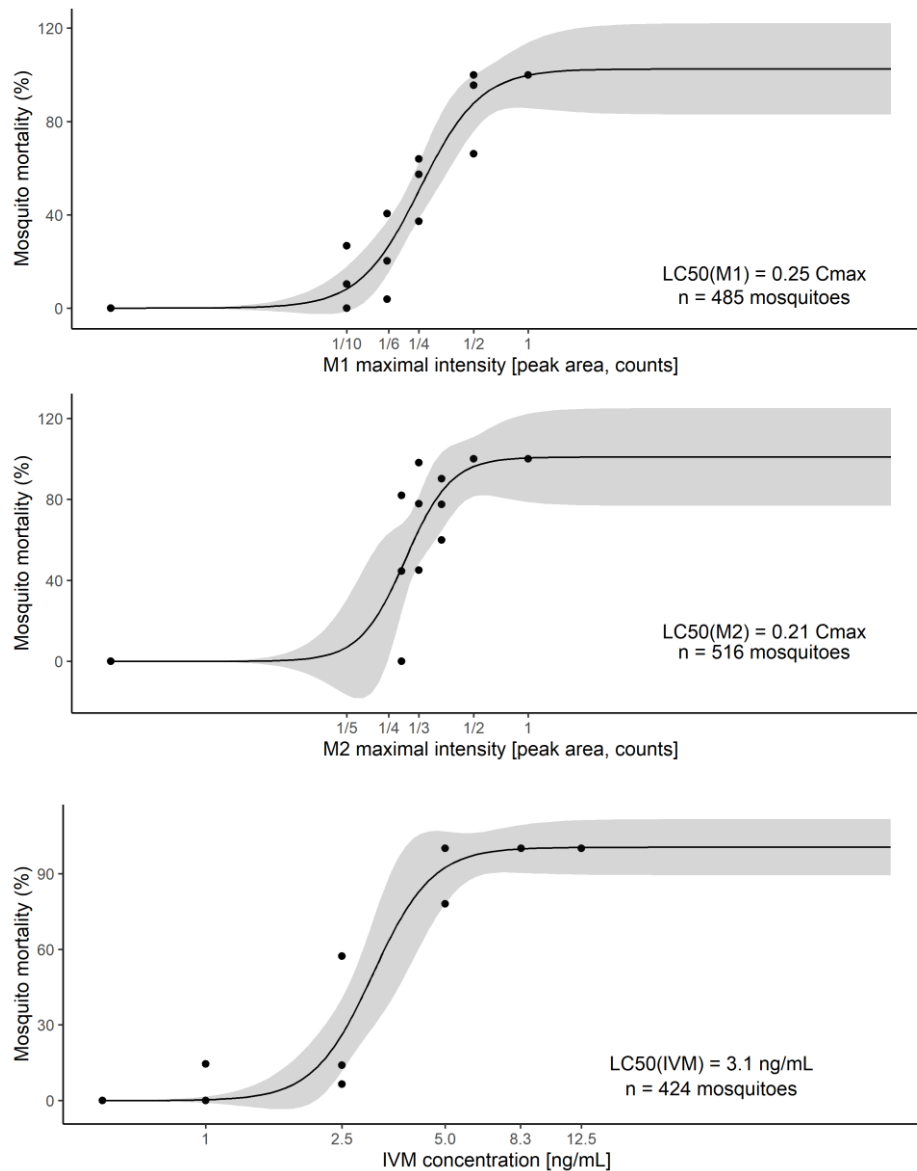
Figure S4 – Compound level above LC$_{50}$ in whole blood for ivermectin (IVM), and metabolites desmethyl-H$_2$B$_{1a}$ (M1) and hydroxy-H$_2$B$_{1a}$ (M2).
The blood samples of the participants of the pharmacokinetics trial (Duthaler et al., 2019), pre- and up to 72h post-ivermectin dose, were analyzed to derive the pharmacokinetic profiles for ivermectin, M1 and M2. The LC$_{50}$ values were computed in **Figure S3** and used to derive the time above LC$_{50}$.

	Time above LC$_{50}$ [h]
M1	69.0
M2	34.0
IVM	69.3