

Elucidation of in vitro chlorinated tyrosine adducts in blood plasma as selective biomarkers of chlorine exposure

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Supporting information

Contents

- S1. Method optimization and validation
- Figure S1. Calibration curves Cl-Tyr and di-Cl-Tyr
- Figure S2. Heatmap of peptides identified with LC-HRMS/MS after pepsin digestion
- Table S1. Extent of protein chlorination after pepsin digestion
- Table S2. Extent of protein chlorination after trypsin digestion
- Table S3. Identified biomarkers for chlorine exposure after trypsin and pepsin digestion.
- Figure S3. Protein coverage of human serum albumin and haptoglobin
- Figure S4. Extracted ion chromatograms of YLYEIAR
- S2. Isotope ratios
- Table S4. Theoretical compared to measured isotope values
- Table S5. Fragmentation pattern of peptide Y(Cl)LYEIAR
- Figure S5. MS/MS spectrum of parent ion K.Y(Cl)LY(Cl)EIAR
- Table S6. Fragmentation pattern of peptide Y(Cl)LY(Cl)EIAR
- Figure S6. MS/MS spectrum of parent ion K.Y(Cl)LY(Cl₂)EIAR
- Table S7. Fragmentation pattern of peptide Y(Cl)LY(Cl₂)EIAR
- Figure S7. Extracted ion chromatograms of HYEGSTVPEKK
- Table S8. Fragmentation pattern of peptide HY(Cl)EGSTVPEKK
- Figure S8. MS/MS spectrum of parent ion HY(Cl₂)EGSTVPEKK.
- Table S9. Fragmentation pattern of peptide HY(Cl₂)EGSTVPEKK

S1. Method optimization and validation

The LC-MS/MS method was optimized for Cl-Tyr (t_r : 6.15 min) and di-Cl-Tyr (t_r : 6.57 min) with $^{13}\text{C}_6$ -3-chloro-L-tyrosine (t_r : 6.14 min) as internal standard. Linear calibration curves were obtained in the range of 1-100 ng/mL with $R^2 = 0.9992$ - 0.9999 (Figure S1). The mean values for the quality controls were within 20% at 1 ng/mL and within 6% relative standard deviation at both 10 ng/mL and 100 ng/mL ($n = 10$, for each concentration). The stability of the analytes was assessed over two weeks and the concentration was within 15% of the nominal value. The sample preparation efficiency of the pronase digest, determined by spiking known concentrations of analyte into human plasma, were respectively $59\% \pm 9\%$ for Cl-Tyr and $56\% \pm 15\%$ for di-Cl-Tyr. The ionization efficiency was slightly improved by the addition of 0.5% formic acid solution of 10 v% in water.

In addition, the influence of the plasma surface area on the effect of the chlorine exposure was examined. It was found that increasing the surface area with a factor of approximately 10 by using the laboratory glass bottle setup instead of a 15 mL polypropylene Corning tube resulted in a 5-7-fold increase in detected Cl-Tyr level in plasma ($n = 3$). Furthermore, it was found that increasing the exposure time from 2 hours to 48 hours did not significantly increase the degree of chlorination (concentrations were within the error range of ± 1 stdv.). This was expected because blood plasma rapidly consumes chlorine. Finally, prolonging the pronase digestion duration beyond 48 hours did not significantly affect the detected levels of Cl-Tyr and di-Cl-Tyr.

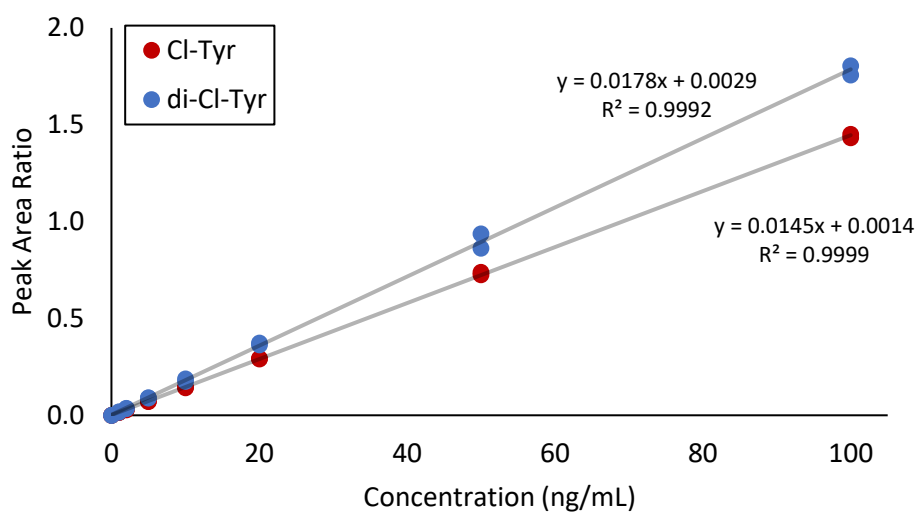


Figure S1. Calibration curves of 3-chlorotyrosine (Cl-Tyr) and 3,5-dichlorotyrosine (di-Cl-Tyr) with internal standard $^{13}\text{C}_6$ -3-chloro-L-tyrosine, detected by LC-MS/MS.

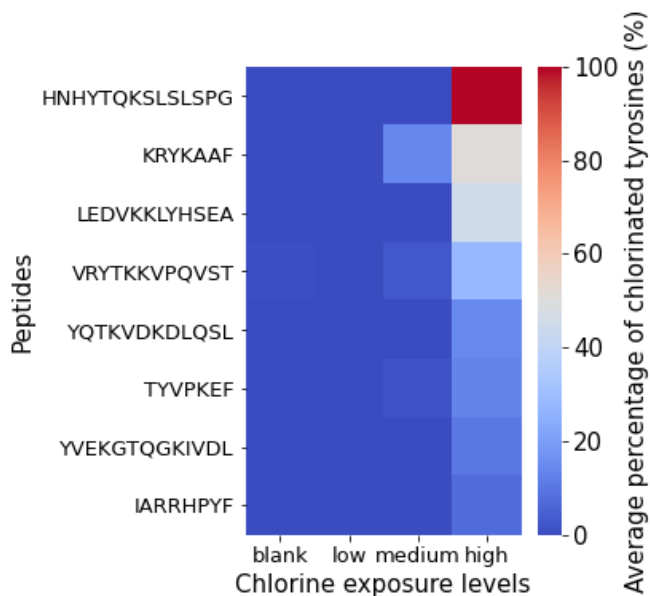


Figure S2. Heatmap of peptides identified with LC-HRMS/MS after pepsin digestion of human blood plasma, with corresponding average percentage of chlorinated tyrosines for non-exposed, low, medium, and high chlorine exposure.

Table S1. Extent of protein chlorination for various chlorine exposure levels after digestion by pepsin (n = 3, \pm SD).

Chlorine exposure level	Average percentage of chlorinated tyrosines (%)
Blank	1.2 \pm 0.4
Low	0.7 \pm 0.6
Medium	3.2 \pm 1.5
High	14 \pm 9

Table S2. Extent of overall protein chlorination for various chlorine exposure levels after digestion by trypsin (n = 3, \pm SD).

Chlorine exposure level	Average percentage of chlorinated tyrosines (%)
Blank	0.2 \pm 0.3
Low	0.5 \pm 0.5
Medium	2.3 \pm 1.8
High	16.2 \pm 2.1

Table S3. Identified biomarkers for chlorine exposure after trypsin and pepsin digestion.

Long peptide	Enzyme	Accession	Protein	Mass	t _R (min)	Cl (#) ^a	N (#) ^b
AHY(CI)GGFTVQNEANK	Trypsin	P02675 FIBB_HUMAN	Fibrinogen beta chain	1568.7	18.5	1	2
AIGY(CI)LNTGYQR	Trypsin	P01023 A2MG_HUMAN	Alpha-2- macroglobulin	1288.6	20.2	1	1
ALSHAVNNY(CI)HK	Trypsin	P04114 APOB_HUMAN	Apolipoprotein B-100	1286.6	15.3	1	2
ASAGLLGAHAAAITAY(CI)	Trypsin	P0COL4 CO4A_HUMAN	Complement C4- A	1490.7	21.6	1	1
ATVLNY(CI)LPK	Trypsin	P01023 A2MG_HUMAN	Alpha-2- macroglobulin	1051.5	21.9	1	3 ^c
AVRPGY(CI)PK	Trypsin	n.a.	n.a.	920.5	15.9	1	4 ^c
DDLY(CI)VSDAFHK	Trypsin	P01008 ANT3_HUMAN	Antithrombin-III	1342.6	20.4	1	3
FSVY(CI)AK	Trypsin	P02765 FETUA_HUMAN	Alpha-2-HS- glycoprotein	846.4	20.3	1	3
GEVPPRY(CI)PR	Trypsin	P02790 HEMO_HUMAN	Hemopexin	1103.5	16.6	1	1
GGTSY(CI)GTGSETESPR	Trypsin	P02671 FIBA_HUMAN	Fibrinogen alpha chain	1605.6	16.3	1	2
GLSVY(CI)ADKPETTK	Trypsin	P02763 A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	1441.7	18.2	1	1
GVALHRPDVY(CI).LLPPAR	Trypsin	P01871 IGHM_HUMAN	Immunoglobulin heavy constant mu	1159.6	19.1	1	2
GY(CI)TQQLAFR	Trypsin	P01024 CO3_HUMAN	Complement C3	1116.5	20.5	1	2
AP.HGPGLIY(CI)R	Trypsin	P02765 FETUA_HUMAN	Alpha-2-HS- glycoprotein	1113.5	19.2	1	1
HNHY(CI)TQKSLSLSPG	Pepsin	P0DOX5 IGG1_HUMAN	Immunoglobulin gamma-1 heavy chain	1601.7	17.4	1	2
R.HPDY(CI)SVVL.LLR	Trypsin	P02768 ALBU_HUMAN	Albumin	1500.8	21.5	1	1
HQLY(CI)IDETVNSNIPTNLR	Trypsin	P02675 FIBB_HUMAN	Fibrinogen beta chain	1307.6	14.9	1	2
HY(CI)EGSTVPEK.K	Trypsin	P00738 HPT_HUMAN	Haptoglobin	1307.6	14.9	1	5
HY(CI2)EGSTVPEK.K	Trypsin	P00738 HPT_HUMAN	Haptoglobin	1341.6	15.1	2	3
YE.IARRHPY(CI)F.Y(CI)APEL	Pepsin	P02768 ALBU_HUMAN	Albumin	1360.6	20.8	2	1
L.IQPDSVVKPY(CI)R	Trypsin	P02675 FIBB_HUMAN	Fibrinogen beta chain	1435.7	18.6	1	2

IY(CI)GNQDTSSQLK	Trypsin	P19823 ITIH2_HUMAN	Inter-alpha- trypsin inhibitor heavy chain H2	1386.6	17.9	1	1
FA.KRY(CI2)KAAF	Pepsin	P02768 ALBU_HUMAN	Albumin	1168.5	19.5	1	2 ^c
FFA.KRY(CI)KAAF	Pepsin	P02768 ALBU_HUMAN	Albumin	1281.6	20.6	1	4 ^c
LEDVKKLY(CI)HSEA	Pepsin	P01009 A1AT_HUMAN	Alpha-1- antitrypsin	1464.7	18.3	1	2
KQL.INDY(CI)V.EK	Trypsin	P01011 AACT_HUMAN	Alpha-1- antichymotrypsin	1041.5	18.7	1	3 ^c
LGEVNTY(CI)AGDLQK	Trypsin	P06727 APOA4_HUMAN	Apolipoprotein A-IV	1440.7	20.0	1	2
LLIY(CI)GASTR	Trypsin	n.a.	n.a.	1026.5	21.0	1	1
NSLFEY(CI)QK	Trypsin	P02671 FIBA_HUMAN	Fibrinogen alpha chain	1061.5	20.6	1	3
EEAPSLR.PAPPPISGGGY(CI)R	Trypsin	P02675 FIBB_HUMAN	Fibrinogen beta chain	1984.0	19.6	1	3
SY(CI)STTAVVTNPK	Trypsin	P02766 TTHY_HUMAN	Transthyretin	1300.6	18.1	1	2
TAQEGDHGSHVY(CI).TK	Trypsin	P01023 A2MG_HUMAN	Alpha-2- macroglobulin	1562.7	14.8	1	3
TY(CI)ETTLEK	Trypsin	P02768 ALBU_HUMAN	Albumin	1017.4	18.2	1	6
SALE.VDETY(CI)VPK	Trypsin	P02768 ALBU_HUMAN	Albumin	1383.6	20.6	1	1
EVDE.TY(CI)VPKE.F	Pepsin	P02768 ALBU_HUMAN	Albumin	1388.6	21.7	1	1
VGPEADKY(CI)R	Trypsin	P02679 FIBG_HUMAN	Fibrinogen gamma chain	1067.5	16.3	1	1
IV.VRY(CI)TKKVPQVST.PTL	Pepsin	P02768 ALBU_HUMAN	Albumin	1438.8	16.3	1	7 ^c
IV.VRY(CI2)TKKVPQVST.PTL	Pepsin	P02768 ALBU_HUMAN	Albumin	1897.0	20.5	1	4 ^c
WY(CI)VDGVEVH.NAK	Trypsin	P01857 IGHG1_HUMAN	Immunoglobulin heavy constant gamma 1	1449.6	16.0	1	4 ^c
Y(CI)AATSQVLLPSK	Trypsin	P01871 IGHM_HUMAN	Immunoglobulin heavy constant mu	1310.7	20.2	1	2
F.Y(CI)APELFFAK	Trypsin	P02768 ALBU_HUMAN	Albumin	1378.7	22.2	1	2
Y(CI)EKPGSPRR	Trypsin	P02751 FINC_HUMAN	Fibronectin	1063.5	15.3	1	1
Y(CI)GAATFTR	Trypsin	P01023 A2MG_HUMAN	Alpha-2- macroglobulin	919.4	18.8	1	2
SI.Y(CI)KPGQTVK	Trypsin	P01023 A2MG_HUMAN	Alpha-2- macroglobulin	1153.6	17.4	1	4

YLQEIY(CI)NSNNQK	Trypsin	P02679 FIBG_HUMAN	Fibrinogen gamma chain	1546.7	19.6	1	1
K.Y(CI)LY(CI)EIAR	Trypsin	P02768 ALBU_HUMAN	Albumin	994.4	21.6	2	3
KK.Y(CI)LYEIAR	Trypsin	P02768 ALBU_HUMAN	Albumin	960.4	20.8	1	4
Y(CI)LY(CI2)EIAR	Trypsin	P02768 ALBU_HUMAN	Albumin	1028.4	21.9	3	1
Y(CI)QQHPGKAPK	Trypsin	P01704 LV214_HUMAN	Immunoglobulin lambda variable 2-14	1186.6	14.3	1	1
Y(CI2)QQKPGKAPK	Trypsin	A0A0C4DH67 KV108_HUMAN	Immunoglobulin kappa variable 1- 8	1594.8	15.2	2	1
Y(CI)QQKPGQAPR	Trypsin	P01619 KV320_HUMAN	Immunoglobulin kappa variable 3- 20	1205.6	14.8	1	5
Y(CI)QTKVDKDLQSL	Pepsin	P02679 FIBG_HUMAN	Fibrinogen gamma chain	1470.7	20.0	1	1
Y(CI)TFELSR	Trypsin	P02774 VTDB_HUMAN	Vitamin D- binding protein	948.4	20.9	1	2
Y(CI)VEKGTQGKIVDL	Pepsin	P01009 A1AT_HUMAN	Alpha-1- antitrypsin	1482.7	20.0	1	1
Y(CI)VGGQEHAH	Trypsin	P02763 A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	1177.5	16.8	1	1

^aNumber of attached chlorine atoms (Cl)

^bPeptides detected in given number of repetitions (N)

^cNot detected in all high chlorine exposure samples

A)



B)

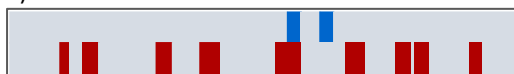


Figure S3. Protein coverage, with sequences identified after pepsin digestion (blue) and trypsin digestion (red). A) Human serum albumin (56% coverage), B) Haptoglobin (33% coverage).

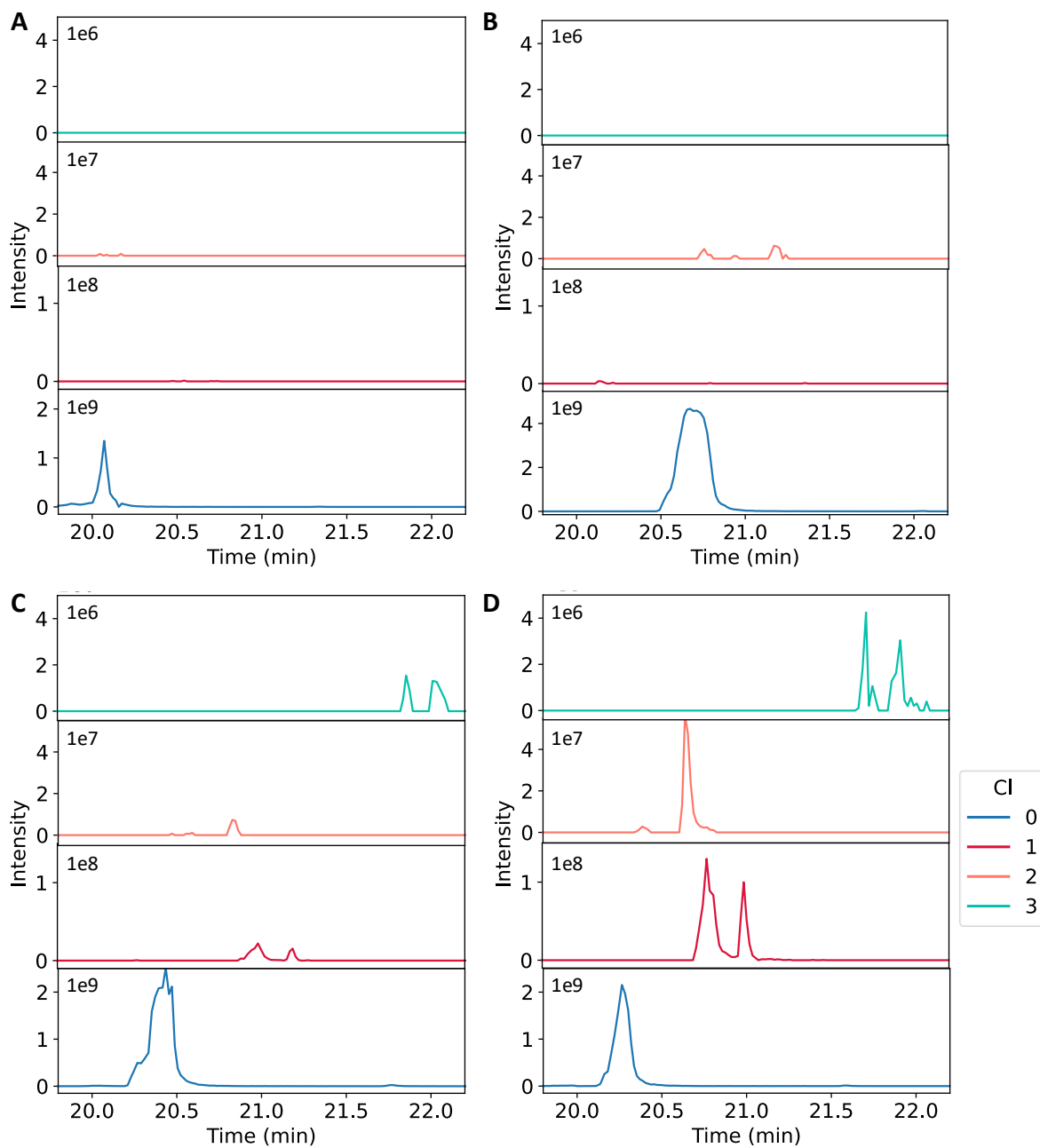


Figure S4. Extracted ion chromatograms of plasma exposed to various chlorine exposure concentrations analyzed by LC-HRMS/MS (Acclaim PepMap C18 column, mobile phase: H₂O (A) and ACN (B) with 0.1% FA), with YLYEIAR at m/z 464.25 and t_R = 20.1-20.7 min (blue), Y(Cl)LYEIAR and YLY(Cl)EIAR at m/z 481.23 and t_R = 20.8-21.0 and 21.0-21.2 min (red), K.Y(Cl)LY(Cl)EIAR at m/z 562.26 and t_R = 20.6-20.8 min (orange), and Y(Cl)LY(Cl)₂EIAR and Y(Cl₂)LY(Cl)EIAR at m/z 515.19 and t_R = 21.7-21.9 and 21.9-22.0 min (green). A) Blank, B) Low exposure, C) Medium exposure, D) High exposure.

S2. Isotope ratios

In the full scan MS spectrum of this doubly charged peptide, a distinct chlorine pattern is visible for single, double and triple chlorination (Figure 6B-D). The unchlorinated peptide showed a single peak as expected (Figure 6A). Because the ^{35}Cl isotope has a natural occurrence of 76% and the ^{37}Cl isotope of 24%, the isotope ratio for the chlorinated peptide can be calculated. When the influence of other isotopes, such as carbon, is also considered, the isotope ratio of a mono-, di- and tri-chlorinated peptide is 2:1 (Figure 6B), 5:4:1 (Figure 6C) and 11:12:5:1 (Figure 6D), respectively.⁴⁹ In Figure 6C, this last peak for the tri-chlorinated peptide was expected at a m/z 518.19, but it was not visible presumably due to its low intensity.

Table S4. Theoretical compared to measured isotope values in full scan MS spectrum analyzed by LC-HRMS/MS of doubly charged chlorinated precursor Y*LY*EIAR.

Chlorination	Theoretical isotope value	Measured isotope value
0	1	1
1	2:1	2:1
2	5:4:1	5:4:1
3	11:12:5:1	11:13:4:0

Table S5. Fragmentation pattern of peptide Y(Cl)LYEIAR with y and b fragments.

#	b (m/z)	Peptide	Y (m/z)	#
1	198.03	Y(+33.96)		7
2	311.12	L	764.43	6
3	474.18	Y	651.35	5
4	603.22	E	488.28	4
5	716.31	I	359.24	3
6	787.34	A	246.16	2
7		R	175.12	1

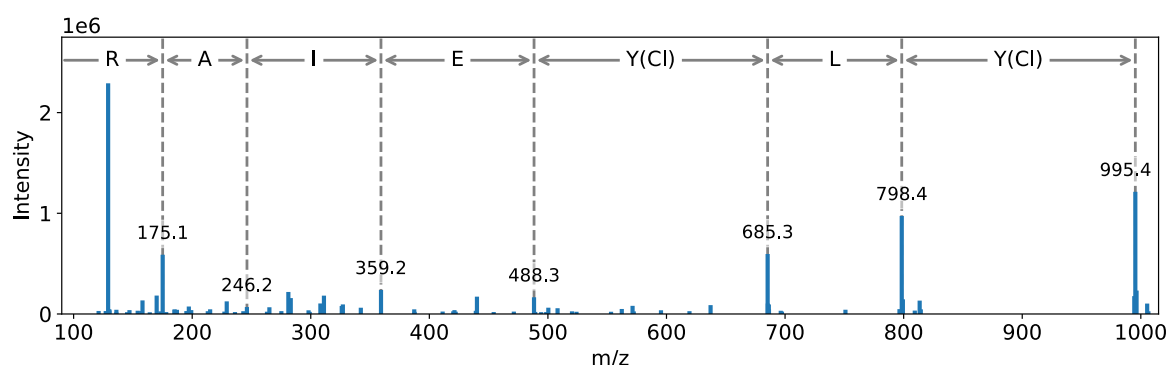


Figure S5. MS/MS spectrum of parent ion K.Y(Cl)LY(Cl)EIAR with m/z 562.262 at an $t_R = 20.64$ min., detected in the trypsin digest of a plasma sample exposed to 70 and 350 mmol/L chlorine gas. The m/z of the γ -fragments and corresponding amino acids are shown.

Table S6. Fragmentation pattern of peptide Y(Cl)LY(Cl)EIAR with y and b fragments.

#	b (m/z)	Peptide	Y (m/z)	#
1	129.10	K		8
2	326.13	Y(+33.96)	995.41	7
3	439.21	L	798.39	6
4	636.24	Y(+33.96)	685.31	5
5	765.28	E	488.28	4
6	878.36	I	359.24	3
7	949.40	A	246.16	2
8		R	175.12	1

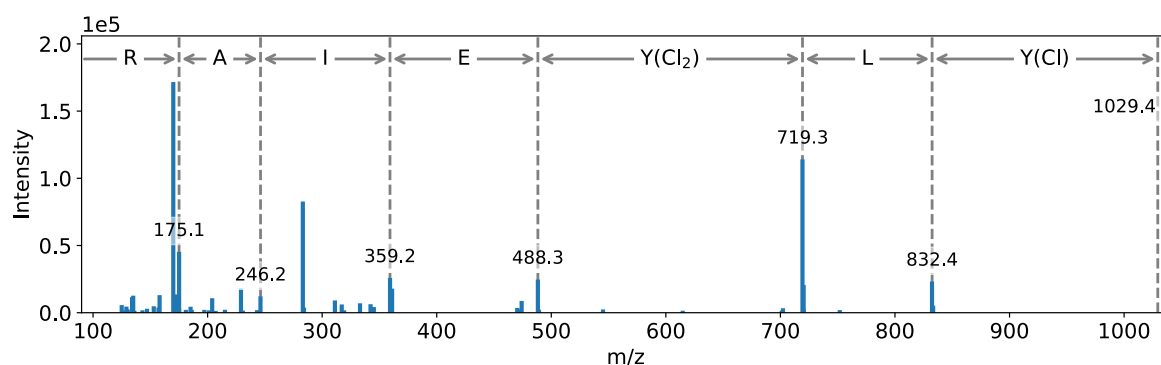


Figure S6. MS/MS spectrum of parent ion Y(Cl)LY(Cl)EIAR with m/z 515.194 at an $t_R = 21.91$ min., detected in the trypsin digest of a plasma sample exposed to 350 mmol/L chlorine gas. The m/z of the y-fragments and corresponding amino acids are shown.

Table S7. Fragmentation pattern of peptide Y(Cl)LY(Cl)₂EIAR with y and b fragments.

#	b (m/z)	Peptide	Y (m/z)	#
1	198.03	Y(+33.96)	1029.4	7
2	311.12	L	832.36	6
3	542.10	Y(+67.92)	719.27	5
4	671.14	E	488.28	4
5	784.23	I	359.24	3
6	855.27	A	246.16	2
7		R	175.12	1

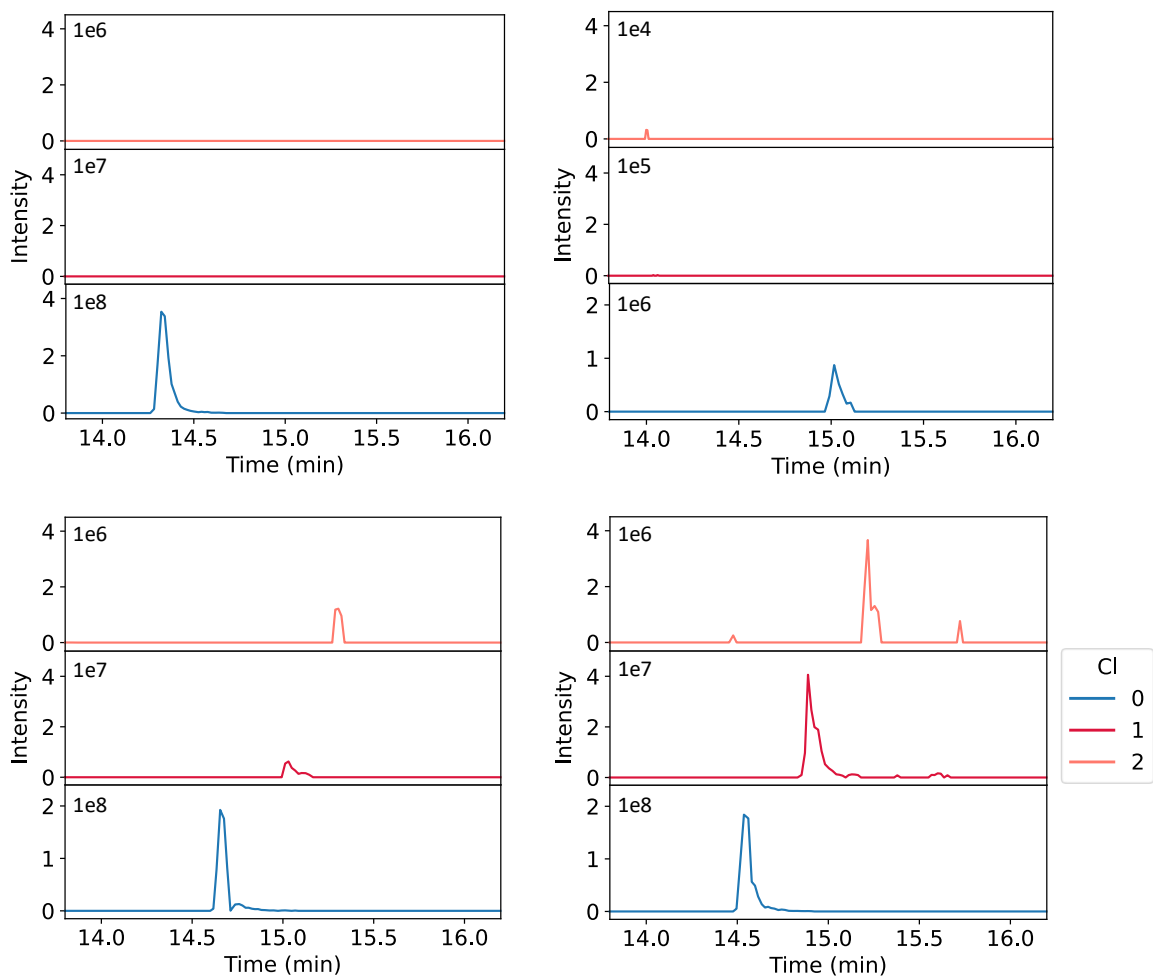


Figure S7. Extracted ion chromatograms of plasma exposed to various chlorine exposure concentrations analyzed by LC-HRMS/MS (Acclaim PepMap C18 column, mobile phase: H₂O (A) and ACN (B) with 0.1% FA), with H₂YEGSTVPEKK at *m/z* 637.823 and *t_R* = 14.3-15.0 min (blue), H₂Y(Cl)EGSTVPEKK at *m/z* 654.805 and *t_R* = 14.9-15.0 min (orange), and H₂Y(Cl)₂EGSTVPEKK at *m/z* 671.785 and *t_R* = 15.2-15.3 min (green). A) Blank, B) Low exposure, C) Medium exposure, D) High exposure.

Table S8. Fragmentation pattern of peptide HY(Cl)EGSTVPEKK with y and b fragments.

#	b	Peptide	y	#
1	138.07	H	1308.60	11
2	335.09	Y(+33.96)	1171.54	10
3	464.14	E	974.52	9
4	521.15	G	845.48	8
5	608.19	S	788.45	7
6	709.24	T	701.42	6
7	808.30	V	600.37	5
8	905.36	P	501.30	4
9	1034.40	E	404.25	3
10	1162.48	K	275.21	2
11		K	147.11	1

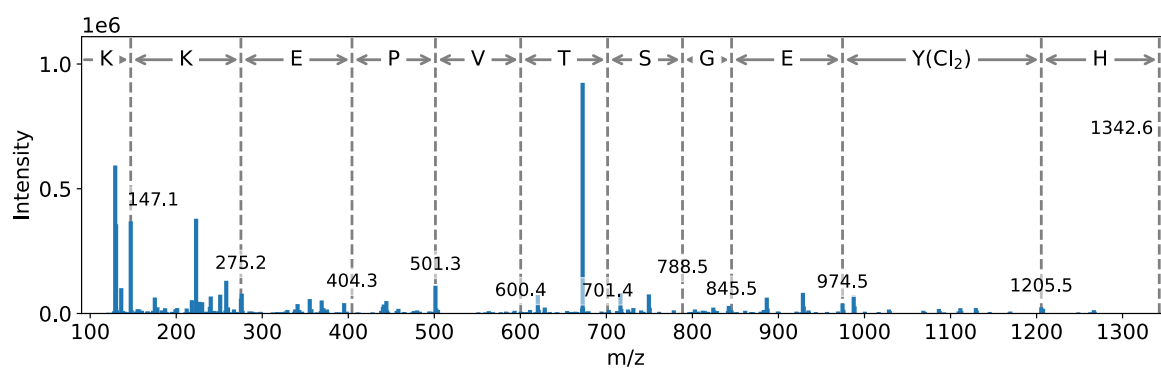


Figure S8. MS/MS spectrum of parent ion HY(Cl₂)EGSTVPEKK with m/z 672.648 at an t_R = 15.21 min., detected in the trypsin digest of a plasma sample exposed to 350 mmol/L chlorine gas. The m/z of the y-fragments and corresponding amino acids are shown.

Table S9. Fragmentation pattern of peptide HY(Cl₂)EGSTVPEKK with y and b fragments.

#	b	Peptide	y	#
1	138.07	H	1342.6	11
2	369.05	Y(+67.92)	1205.5	10
3	498.09	E	974.52	9
4	555.12	G	845.47	8
5	642.15	S	788.45	7
6	743.20	T	701.42	6
7	842.26	V	600.37	5
8	939.32	P	501.30	4
9	1068.4	E	404.25	3
10	1196.5	K	275.21	2
11		K	147.11	1