

# LC-ESI MS/MS detection of the alkylated dipeptide HETE-CP to prove sulfur mustard exposure: application to plasma samples provided by the *Organisation for the Prohibition of Chemical Weapons*

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**Aims:** The chemical warfare agent sulfur mustard (SM) still represents a serious threat for civilians and military personnel especially in terroristic scenarios and by accidental exposure while weapons destruction. SM is banned by the Chemical Weapons Convention which adherence is controlled by the Organisation for the Prohibition of Chemical Weapons (OPCW). In this context, bioanalytical procedures for verification of exposure to SM are inevitable demands according to the international and criminal law and are thus evaluated by the OPCW. As SM alkylates diverse endogenous proteins to hydroxy-ethylthioethyl (HETE)-adducts, these molecules are targets of modern instrumental analysis. Accordingly, aim of this study was to develop a novel procedure to prove the presence of albumin-SM adducts in plasma. **Methods:** The human albumin adduct (alkylation of cysteine residue, C<sup>34</sup>) was isolated from plasma by affinity extraction and proteolytically cleaved by pronase to detect the alkylated dipeptide HETE-CysPro (HETE-CP) by  $\mu$ LC-ESI MS/MS. Five qualifier product ions were monitored including the most intense diagnostic signals at m/z 105 and m/z 137. **Results and Discussion:** Current lots of commercially available pronase produced HETE-CP instead of the expected HETE-CPF tripeptide. Variations of chromatographic temperature documented a dynamic on-column equilibrium of cis- and trans-configuration at the imide bond between cysteine and the C-terminally bound proline in HETE-CP. Chromatography at 50°C revealed one narrow peak ideal for sensitive detection. Microbore analysis reveals a cycle time of 60 min at 18 % ion suppression. The amount of HETE-CP was directly proportional to the concentration of SM added to plasma yielding a sufficient lower limit of detection (LOD) corresponding to 50 nM SM. **Conclusion:** The  $\mu$ LC-ESI MS/MS method is reliable for detection of plasma albumin-derived adducts of SM with a LOD corresponding to 50 nM SM in plasma. The entire procedure was successfully applied to samples of a real accidental SM exposure and to samples sent by the OPCW during the course of the 5<sup>th</sup> biomedical exercise (5. BME).

## 1. Introduction

Chemical warfare agents (CWA) especially organophosphorus nerve agents (e.g. tabun, sarin, soman, VX) and vesicants (e.g. sulfur mustard, SM) are banned by the Chemical Weapons Convention (CWC). The CWC interdicts development, production, transport and use of CWA. Adherence of these international rules is controlled by the Organisation for the Prohibition of Chemical Weapons, OPCW [1] honored with the Nobel Peace Prize in 2013. The OPCW also operates an international network of analytical laboratories qualified for CWA detection in environmental and biological samples to verify an alleged use of such agents. To train and test bioanalytical skills of relevant laboratories biomedical exercises (BME) are exe-

cuted periodically. In February 2015 the 5. BME requisitioned detection of SM-exposure in plasma samples by means of its protein adducts. Protein adducts are covalent reaction products of CWA and endogenous proteins characterized by high stability and longer half-lives of several weeks up to months *in vivo* [2-5]. Therefore, such targets provide properties most suitable for retrospective analysis of poisoning (verification analysis) especially if sample drawing from humans was delayed by some days [6].

Adducts of SM with hemoglobin [7,8] and human albumin (HSA) [8,9] have proven their applicability and reliability to verify SM-poisoning. Fundamental procedures presented by Noort et al. comprised albumin adduct extraction from plasma followed by pronase-catalyzed cleavage to produce the hydroxy-ethylthioethyl derivative of the tripeptide CysProPhe (HETE-CPF) accessible by liquid-chromatography-electrospray tandem mass spectrometry (LC-ESI MS/MS) [8,9].

When trying to set up that method in our laboratory we failed in the detection of the tripeptide but found the corresponding alkylated dipeptide HETE-CP nearly exclusively instead. Despite its non-favourable chromatographic properties (elution as a very broad peak from C18 material under standard conditions at 30°C) we optimized separation and detection of HETE-CP and thus established a novel sensitive and reliable procedure for verification of SM-exposure. Accordingly, this technique was successfully applied to diverse relevant real samples. Results obtained for plasma samples provided by the OPCW within the course of the 5. BME are partly presented here.

## 2. Material and Methods

### 2.1. Chemicals

SM and 8-fold deuterated SM (d8-SM) were made available by the German ministry of defence and tested for integrity and purity in-house by NMR. Human EDTA plasma was purchased from Dunn Labortechnik (Asbach, Germany), formic acid (FA,  $\geq 98\%$  p.a. ACS) from Carl Roth (Karlsruhe, Germany) and  $\text{NH}_4\text{HCO}_3$  (Ultra grade,  $\geq 99.5\%$ ) from Fluka (Buchs, Switzerland). Pronase from *Streptomyces griseus* was delivered by Roche (Mannheim, Germany, lot No. 70327222). Water (HPLC grade), acetonitrile (ACN, gradient grade), and iso-propanol (iPrOH, GC grade) were obtained from Merck (Darmstadt, Germany) and deuterated atropine (d3-atropine) from CDN Isotopes (Pointe-Claire, Quebec, Canada).

### 2.2. Incubation, extraction and enzymatic cleavage of HSA from plasma

A solution of SM (60  $\mu\text{l}$  in iPrOH) or d8-SM (60  $\mu\text{l}$  in iPrOH) was mixed with albumin solution (1440  $\mu\text{l}$ , 40 mg/ml) or EDTA plasma (1440  $\mu\text{l}$ ) for 2 h incubation at 37°C under gentle shaking. Until further processing incubation mixtures were stored at  $-25^\circ\text{C}$ , if necessary. Vesicant concentrations in plasma ranged from 0.05 to 100  $\mu\text{M}$ .

According to John et al. [4,10] HSA was extracted from plasma using *albumin removal columns* (ProteoExtract, Calbiochem, Merck, Darmstadt, Germany) and eluates were concentrated by ultrafiltration (UF, molecular weight cut-off, MWCO, 10 kDa, Vivaspin 500 centrifugal concentrator, Satorius Stedim, Göttingen, Germany) to 100  $\mu\text{l}$ . The retentate was washed twice with  $\text{NH}_4\text{HCO}_3$  buffer (600  $\mu\text{l}$ , 50 mM) by UF (MWCO 10 kDa) and remaining retentate (100  $\mu\text{l}$ ) was diluted with the same buffer (100  $\mu\text{l}$ ).

For proteolytic cleavage of HSA pronase was added (100  $\mu\text{l}$ , 10 mg/ml in 50 mM  $\text{NH}_4\text{HCO}_3$ ) following incubation for 2 h at 37°C under gentle shaking. Subsequent to UF (MWCO 10 kDa), the retentate (100  $\mu\text{l}$ ) was washed twice with 100  $\mu\text{l}$   $\text{NH}_4\text{HCO}_3$  buffer as described

above. Resulting filtrates were combined (400  $\mu$ l) and diluted 1:3 with 0.5 % (v/v) FA containing 3 ng/ml d3-atropine as internal standard for  $\mu$ LC-ESI MS/MS analysis.

### 2.3. OPCW plasma samples

EDTA plasma samples of the 5. BME sent by the OPCW in February 2015 were extracted (100  $\mu$ l), concentrated, enzymatically cleaved and analyzed by  $\mu$ LC-ESI MS/MS as described herein for qualitative detection of HETE-CP as a biomarker of SM exposure in plasma.

### 2.4. $\mu$ LC-ESI MS analysis for verification

The  $\mu$ LC system comprised of a 1431 MicroPro pump (Eldex Laboratories, Napa, CA, USA) an Edurance autosampler and Mistral column oven (Spark Holland, Emmen, The Netherlands), and a Degasy Populaire degasser (Sunchrom, Friedrichsdorf, Germany). The autosampler was controlled by Endurance/Midas 3.10 (SCPA) and pumps by the MicroPro 1.0 software (SCPA, Weyhe-Leeste, Germany). Chromatography was on-line coupled to a 4000 QTrap mass spectrometer (ABSciex, Darmstadt, Germany) controlled by the Analyst 1.6 software.

Separation of 20  $\mu$ l sample was done at 50°C on an Atlantis T3 column (150 x 1.0 mm I.D., 3  $\mu$ m, 100 A, Waters) protected by a precolumn (security guard cartridges, widepore C18 4 x 2 mm I.D., Phenomenex) at a flow of 30  $\mu$ l/min using solvent A (0.05 % v/v FA) and solvent B (ACN/H<sub>2</sub>O 80:20 v/v, 0.05 % v/v FA) in the following gradient mode: t[min]/B[%]: 0/0, 38/40, 39/80, 43/80, 44/0, 45/0. Initial equilibration was done for 15 min under starting conditions and the solvent reservoir was kept at 40°C.

Mass spectrometric detection was carried out after positive ESI in multiple reaction monitoring mode (MRM). The following settings were used: curtain gas (CUR) 30 psi ( $2.07 \cdot 10^5$  Pa), ionization voltage (IS) 4500 V, temperature 200°C, declustering potential (DP) 60 V (HETE-adduct) and 86 V (d3-atropine), entrance potential (EP) 10 V, cell exit potential (CXP) 10 V, heater gas (GS1) and turbo ion spray gas (GS2) both 50 psi ( $3.45 \cdot 10^5$  Pa), and dwell time 90 ms. The following transitions were recorded for HETE-CP from m/z 323.1 to the qualifying ions at m/z 105.0; 116.2; 137.0; 200.1, and 217.3 using a unique collision energy (CE) of 25 V, except for the transition to m/z 116.2 (CE 30 V). The deuterated analogue d8-HETE-CP was monitored by transitions from m/z 331.1 to m/z 145.0 and m/z 113.0 at CE 25 V and d3-atropine was recorded by transitions from m/z 293.2 to m/z 93.2 and m/z 127.1 at CE 35 V.

### 2.5. Temperature-dependent analysis of HETE-CP isomers by LC-ESI MS/MS

Temperature-dependent elution profiles of HETE-CP (derived from pure HSA incubated with SM) were analyzed using a narrowbore LC system consisting of an UltiMate 3000 Standard LC System (Dionex, Sunnyvale, CA, USA) including an UltiMate 3000 pump, autosampler, and column compartment that was on-line coupled to the 4000 QTrap mass spectrometer described above. Separations were done on an Atlantis T3 column (150 x 2.1 mm I.D., 3  $\mu$ m, 100 A, Waters, Eschborn, Germany) connected with a precolumn (security guard cartridges, widepore C18 4 x 2 mm I.D., Phenomenex, Aschaffenburg, Germany) at a flow of 200  $\mu$ l/min after injection of 20  $\mu$ l. Gradient elution was carried out using solvent A (0.1 % v/v FA) and solvent B (ACN/H<sub>2</sub>O 80:20 v/v, 0.1 % v/v FA): t[min]/B[%]: 0/10, 13/50, 14/85, 17/85, 18/10, 20/10 after 1 min equilibration under starting conditions. Temperature of the column and solvent reservoir was adjusted to 1, 10, 15, 20, 23, 30, 40 and 50°C, respectively. The MS operated under the following conditions: CUR 50 psi ( $3.45 \cdot 10^5$  Pa), IS 4500 V, temperature

300°C, DP 60 V, EP 10 V, CXP 10 V, GS1 and GS2 both 60 psi ( $4.14 \times 10^5$  Pa), and dwell time 90 ms. MRM transitions of HETE-CP were as mentioned above. MS data analysis and control of the mass spectrometer was performed with Analyst 1.6 software and a Dionex chromatography MS link (version 2.12.0.3414).

### 3. Results and Discussion

#### 3.1. Identification of the HETE-CP dipeptide as a marker for SM exposure

HSA extraction from plasma using albumin removal columns yielded good recoveries of 90 % and highest purity as proved by gel electrophoresis (data not shown) [4,10]. Noort et al. reported on a verification procedure for SM-exposure based on LC-ESI MS/MS detection of the alkylated tripeptide HETE-CPF derived from HSA [8,9]. However, current lots of pronase – representing a complex enzyme mixture of about 10 endo- and exopeptidases- provided by common suppliers showed different activity resulting in the production of the alkylated dipeptide HETE-CP nearly exclusively (Fig. 1). As this product has been shown to be stable against further proteolytic degradation [4] we developed a novel method targeting HETE-CP appropriate for sensitive detection and thus for reliable verification analysis.

**D<sup>1</sup>AHKSEVAHRFKDLGEENFKALVLIIFAQYLQQCFEDHVKLVNEVTEFAKT**

enzymatic cleavage by pronase

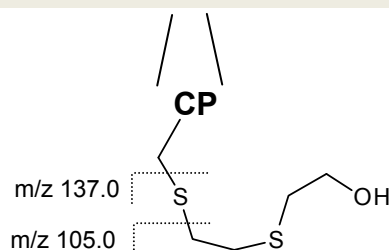


Fig. 1. Pronase-catalyzed cleavage of HSA (UniProt acc. No. P02768) alkylated at Cys<sup>34</sup> by sulfur mustard yielded the diagnostic hydroxy-ethylthioethyl-derivative of the dipeptide CysPro (HETE-CP) accessible for  $\mu$ LC-ESI MS/MS detection [4]. N-terminal sequence of albumin is illustrated without signal- and propeptide from D<sup>1</sup>-T<sup>52</sup>. Structure of HETE-CP also depicts the sulfur containing side chain of Cys<sup>34</sup>. Fragment ions monitored by  $\mu$ LC-ESI MS/MS (m/z 105.0 and 137.0) are indicated at their cleavage sites.

HETE-CP and d8-HETE-CP derived from pure HSA incubated with 100  $\mu$ M of the vesicants yielded characterizing CID spectra after  $\mu$ LC-ESI MS/MS analysis in the product ion scan mode (Fig. 2).

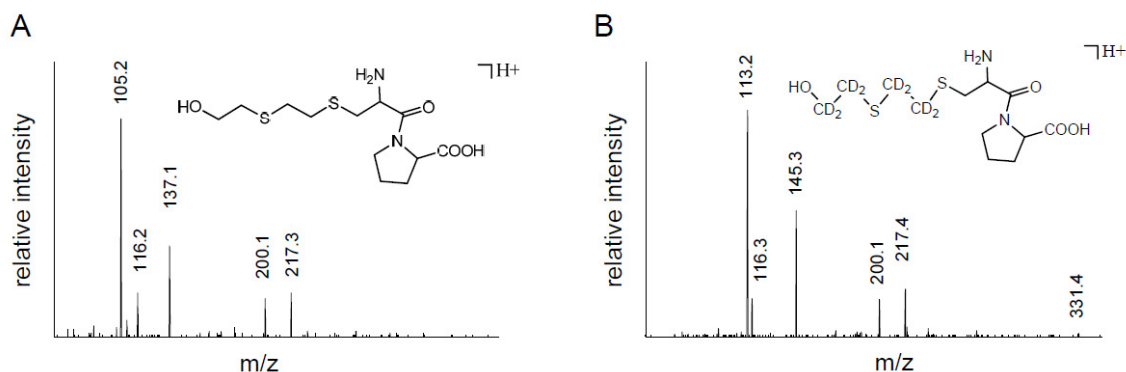


Fig. 2. MS/MS spectra of HETE-CP (A) and its eight-fold deuterated analogue d8-HETE-CP (B) after positive electrospray ionization and collision-induced dissociation.

Product ions comprised most intense diagnostic fragments unambiguously indicating the presence of alkylation by a hydroxy-ethylthioethyl moiety bound to the side chain thiol-group of a cysteine residue. As indicated in Figure 1 the signal at  $m/z$  105.2 ( $m/z$  113.2 for d8-HETE-CP) corresponded to the single charged hydroxy-ethylthioethyl moiety and the signal at  $m/z$  137.1 ( $m/z$  145.3 for d8-HETE-CP) to the same fragment prolonged by the sulfur atom of the cysteine side chain.

### 3.2. Characterization of on-column cis/trans isomerization of HETE-CP

On conventional reversed-phase C18 material at an usual chromatographic temperature of 30°C a very broad peak was obtained for HETE-CP not favourable for sensitive detection [4,9]. Therefore, we tried to optimize the elution profile by choosing other stationary phases and changing the gradient (data not shown). On the one hand we succeeded in peak sharpening but on the other hand we could not avoid a permanent peak splitting potentially indicating the presence of two isobaric compounds. In analogy to an earlier study investigating the 11-hydroxy-thromboxane B<sub>2</sub> anomers equilibrium [11,12] we varied the chromatographic temperature of a narrowbore HPLC system to elaborate the effect on dipeptide elution (Fig. 3). It was found that HETE-CP underwent an on-column equilibrium reaction between a cis- and a trans-isomer due to the imide-bond of the C-terminal proline residue (Fig. 3, chromatogram at 1°C). Elevated chromatographic temperatures (50°C) enabled to obtain a narrow peak appropriate for sensitive detection (Fig. 3). Therefore, 50°C was set for the verification analysis using solvent reservoirs kept at 40°C.

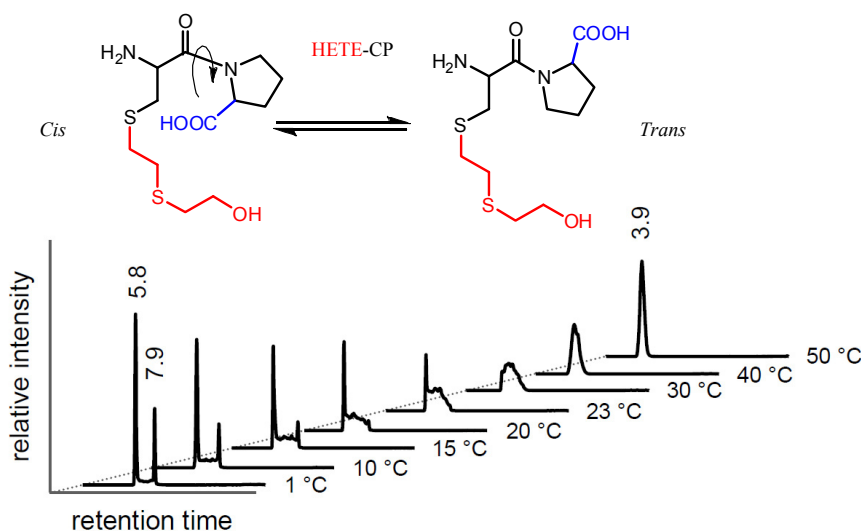


Fig. 3. Temperature-dependent cis/trans isomerization of the alkylated dipeptide HETE-CP derived from an adduct of HSA and SM after enzymatic cleavage with pronase. HETE-CP was chromatographed at indicated temperatures on an Atlantis T3 column (150 x 2.1 mm I.D., 3 mm, 100 Å) at 200 µl/min using solvent A (0.1 % v/v FA) and solvent B (ACN/H<sub>2</sub>O 80:20 v/v, 0.1 % v/v FA) in the following gradient mode: t[*min*]/B[%]: 0/10; 13/50; 14/85; 17/85; 18/10; 20/10 with an initial 1 min equilibration period under starting conditions. Detection was done in the multiple reaction monitoring mode, MRM, ( $m/z$  323.1 >  $m/z$  105.0 is illustrated). Total peak areas were independent on the temperature applied (RSD 3.2%) indicating analyte stability.

### 3.3. µLC-ESI MS/MS for verification

Based on results described above we established a robust µLC-ESI MS/MS method suitable for sensitive and selective detection of HETE-CP. Blank plasma did not cause any interferences at the retention time of the analyte,  $t_R$  20.1 min (Fig. 4 A). The lower limit of detection (LOD) based on the  $m/z$  105.0 transition corresponded to 50 nM SM in human

EDTA plasma sufficient to detect blister-generating SM concentrations. The cycle time (duration between two injections) was 60 min and thus quite long. Therefore, we also developed a much faster but slightly less sensitive method (LOD corresponding to 100 nM SM) operating with narrowbore dimension (cycle time 16 min, data not shown) [4]. Nevertheless, the microbore method was successfully applied to document real cases of accidental SM exposure in humans and proved its reliability for samples of the OPCW sent within the course of the 5. BME (Fig. 4 C).

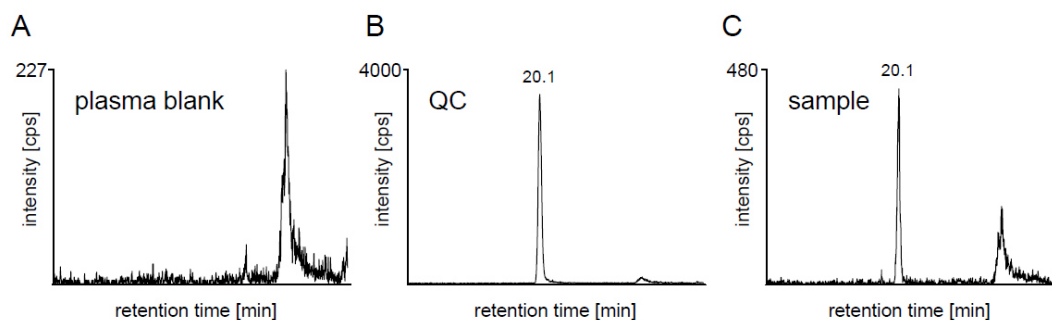


Fig. 4. Extracted ion chromatograms of HETE-CP ( $m/z$  323.1 >  $m/z$  105.0) A) human EDTA plasma blank, B) quality control sample (QC) of 10  $\mu$ M SM in human EDTA plasma, C) plasma sample of 5. BME sent by the OPCW. Chromatographies of 20  $\mu$ l sample (corresponding to a 1.7  $\mu$ l plasma equivalent) were performed on an Atlantis T3 (150 x 1.0 mm I.D., 3  $\mu$ m, 100 A) column in gradient mode using solvent A (0.05 % v/v FA) and solvent B (ACN/H<sub>2</sub>O 80:20 v/v, 0.05 % v/v FA):  $t$ [min]/B[%]: 0/0; 38/40; 39/80; 43/80; 44/0; 45/0 including an initial 15 min equilibration period under starting conditions.

### 3.4. Analysis of 5. BME samples provided by the OPCW

Verification analysis requires unambiguous identification of the analyte what can be achieved by e.g. determination of the relative retention time (RRT) as well as detection of as many product ions as possible and reasonable. Accordingly, we used the  $\mu$ LC-ESI MRM method for most sensitive and selective detection of HETE-CP monitoring the 5 most intense fragments at  $m/z$  217.3, 200.1, 137.0, 116.2, and 105.0.

RRT was calculated in comparison to d3-atropine,  $t_R(\text{d3-atropine})/t_R(\text{HETE-CP})$ , and found to be 1.25 in quality control samples. RRT of all 6 samples of the 5. BME did not differ by more than 0.8 % of that reference value indicating detection of the targeted molecule.

Tab. 1.  $\mu$ LC-ESI MS/MS analysis of a QC and a sample of the 5. BME.

ion>transition [m/z]	sample		QC		criteria	
	area [cts]	ion ratio	area [cts]	ion ratio	tolerance	result
323.1 > 105.0	36600	100 %	178000	100 %	-	OK
323.1 > 137.0	18400	50.3 %	92500	52 %	52 $\pm$ 10.4 (%)	OK
323.1 > 217.3	5820	15.9 %	21700	12.2 %	12.2 $\pm$ 3.7 (%)	OK
323.1 > 200.1	4760	13.0 %	24500	13.8 %	13.8 $\pm$ 4.1 (%)	OK
323.1 > 116.1	6560	17.9 %	25300	14.2 %	14.2 $\pm$ 4.3 (%)	OK

Structure confirmation was done by calculating the fragment ion ratios that had to fit defined reference values obtained from quality control samples (QC) analyzed before (Fig. 4 B).

These ion ratios were deduced from the peak area of each transition compared to the peak area of the most intense signal at  $m/z$  105 set to 100%. The method-dependent and HETE-CP specific values and tolerance intervals (according to the OPCW guidelines) of a standard as well as of one of the OPCW samples are listed in Table 1. Results document reliability and high selectivity of the method.

#### 4. Conclusions

We herein present the development of a novel  $\mu$ LC-ESI MS/MS method for verification analysis of human plasma to prove exposure to SM [4]. The sample preparation procedure considers current lots of commercially available pronase that exhibit a different enzyme activity/specificity than earlier lots used by other groups [8,9]. Furthermore, we characterized the on-column isomerism of *cis/trans* HETE-CP causing tremendous peak broadening hampering sensitive detection. Use of an elevated chromatographic temperature of 50°C resulted in a narrow peak for HETE-CP elution. Monitoring 5 fragment ions after CID supported highly selective analysis allowing structure confirmation by their corresponding ion ratios. The entire procedure was reliable and proved its applicability for real life and training samples delivered by the OPCW.

#### 5. References

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