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ORIGINAL ARTICLE

A rapid and robust assay for the determination of the amino acid hypusine as a possible biomarker for a high-throughput screening of antimalarials and for the diagnosis and therapy of different diseases

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Abstract Eukaryotic initiation factor 5A (eIF5A) has recently been identified as a biomarker of prognostic significance and therapeutic potential for the treatment in hepatocellular carcinoma. This prompted us to establish a rapid and robust assay to determine deoxyhypusine and hypusine formed with the purified enzymes deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH) from *Plasmodium* to develop a rapid screening assay for antimalarial drugs. The peptide hydrolysate obtained from hypusinylated eIF5A was analyzed by ultra performance liquid chromatography (UPLC) with retention times for deoxyhypusine of 7.44 min and for hypusine of 7.30 min, respectively. The limit of detection for both compounds was 0.144 ng/µl. Determination of the specific activity of *Plasmodium* DOHH resulted in a twofold higher specific activity than its human counterpart. Following the iron-complexing strategy of the ferrous iron which is present in the active site of *Plasmodium* DOHH, a series of iron chelating compounds was tested. 2,2′-Dipyridyl and mimosine abolished DOHH activity completely while 4-oxo-piperidine-carboxylates i.e. the nitrophenylether JK8-2 and EHW 437, the oxime ether of the piperidine aldehyde, showed no inhibition although they were highly active in in vitro cultures of *Plasmodium* and in vivo in a rodent mouse model. The method allows a high-throughput screening (HPTS) of antimalarial drugs and the evaluation of eIF5A as a biomarker.

Keywords Ultra performance liquid chromatography · Hypusine · *Plasmodium* · Analytics of a biomarker

Abbreviations

eIF5A	Eukaryotic initiation factor 5A
UPLC	Ultra performance liquid chromatography
HPTS	High-throughput screening
DHS	Deoxyhypusine synthase
DOHH	Deoxyhypusine hydroxylase
AccQ-Tag	6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate
2ME	2-Mercaptoethanol
OPA	*ortho*-Phthalaldehyde
PDA	Photodiode-array detector

Introduction

During the last years there has been a considerable shift in the necessities and demands of the healthcare system worldwide. These shifts are due either to chronic diseases and ageing demographics or infectious diseases in the developing world.

In the new world malaria still belongs to the sixth most important infectious diseases (World Health Organization

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2008). By contrast in the old world, in Europe, 3.2 million Europeans are diagnosed with cancer, mostly breast, colorectal or lung cancers (http://ec.europa.eu/health-eu/health_problems/cancer/index_de.htm). To implement personalized medicine, effective tests predictive of response to treatment or susceptibility are needed. Appropriate biomarkers are necessary to guarantee individual treatments for a patient's personalized medicine (Vegvari and Marko-Varga 2010).

The novel amino acid hypusine, a *N*-hydroxybutyllysine derivative is present in eukaryotic initiation factor 5A (eIF5A) which is the only known cellular protein to contain this posttranslational modification. Although many different functions have been attributed to this protein, eIF5A has recently been shown to be involved in translation elongation (Saini et al. 2009; Gregio et al. 2009) rather than in translation initiation. The hypusine residue is not only essential for cell proliferation in eukaryotes but also for eIF5A activity. Hypusine biosynthesis is performed within two sequential steps catalyzed by deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH). In the first step DHS transfers the aminobutyl moiety from the triamine spermidine to an ε-amino group present in lysine of the hypusine loop in eIF5A. DOHH completes hypusine formation by hydroxylation. Hypusine modification plays a significant role in proliferation of different diseases i.e. cancer (Balabanov et al. 2007), infectious diseases (Moritz et al. 2004), and in diabetes (Maier et al. 2010). Recently eIF5A was identified as a biomarker with prognostic significance in therapy in hepatocellular carcinoma (Lee et al. 2010). The authors used a comparative proteomic and genomic approach resulting in up-regulation of the human isoform eIF5A2 playing the essential role in tumour venous infiltration in hepatocellular carcinoma. Thus its evaluation as a predictive, prognostic and pharmacodynamic biomarker is a challenging area for analytical standardization of this posttranslational modification.

Hitherto, hypusination of eIF5A has only been analyzed radioactively by a conventional filter assay with [^{14}C]-spermidine incorporation (Kang et al. 1995). This assay is rather inaccurate since non-incorporated radioactivity cannot be completely washed away from the filter (Kang et al. 1995). An alternative method was performed by HPLC coupled to fluorescence detection with *o*-phthalaldehyde for derivatizing the primary amino groups of the hypusine peptide hydrolysate. *N*-Epsilon-(5-aminopentyllysine) which is a structural analogue of deoxyhypusine was used as an internal standard. This separation resulted in retention times of deoxyhypusine with 17.2 min and for hypusine with 14.4 min, respectively (Beppu et al. 1996) dependent on the gradient being used. However, reproduction of these experiments proved that the imine derivatives showed non-reproducible stabilities (Kaiser et al. unpublished).

We have recently identified DOHH from *P. falciparum* which completes the hypusine pathway by hydroxylation of deoxyhypusinylated eIF5A (Frommholz et al. 2009; Kerscher et al. 2010). Deoxyhypusine and hypusine analysis was performed by GC/MS after hydrolysis of the hypusine modification and methyl chloroformate derivatization. However, no quantification of either deoxyhypusine or hypusine was possible. Since DOHH from *Plasmodium* has very peculiar features which are not present in the human counterpart it might be a valuable drug target which has to be validated.

Recently, cellular polyamines have been separated by HPLC after AccQ-Tag derivatization (Lee et al. 2009). This prompted us to develop an alternative assay to quantify the intermediate deoxyhypusine and the final product hypusine by ultra performance liquid chromatography (UPLC).

Application of UPLC enabled us to detect deoxyhypusine in a peptide hydrolysate within a retention time of 7.44 min after derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ-Tag, Waters Germany) while hypusine had a retention time of 7.3 min.

These data will provide the basis to develop a rapid and robust assay for a high-throughput screening (HPTS) of novel drugs inhibiting the biosynthetic enzymes of the hypusine pathway.

Materials and methods

Expression of the *eIF5A*, *dhs*, *dohh* genes in pET-15b and pET-28a vectors in *E. coli* BL21 (DE3) cells and subsequent purification by nickel-chelate chromatography

E. coli BL21 (DE3) cells containing the recombinant *eIF5A and dhs* plasmid were grown for expression with pET-15b vector in LB medium containing ampicillin 30 µg/ml. The recombinant *dohh* plasmid for expression was pET-28a and was grown in LB medium containing kanamycin 15 µg/ml. One sample from the expressing strain was taken and centrifuged at 13,000 rpm for 2 min. Cells were lysed with 400 µl lysis buffer (50 mM Tris–HCl, pH 8.0, 2 mM EDTA), centrifuged and again resuspended in lysis buffer and sonicated twice at 4°C for 30 s (tip 1 at 50% using a Branson sonifier). After centrifugation for 10 min at 16,000 rpm at 4°C, samples were diluted 1:1 in loading buffer (20 mM Tris, pH 6.8, 2% (w/v) SDS, 2 mM EDTA, 20% (V/V) glycerol, 0.3% bromophenolblue) heated at 100°C and run on a 10% SDS polyacrylamide gel at 100 V.

Protein purification was performed by nickel-chelate affinity chromatography under native conditions, according to the manufacturer's (Qiagen) protocol.

DHS assay

DHS activity was determined in a reaction mixture of 1 ml containing spermidine, eIF5A from *P. vivax* (40 µM each) or human, 0.5 mM NAD^+ and purified DHS protein from *Plasmodium* or human (Lee et al. 2001) obtained by nickel-chelate chromatography. The incubation was performed at 30°C for 2 h. The unmodified and modified eIF5A precursor protein was recovered by a Microcon-YM 100 kDa column (Amicon, Millipore, Schwalbach, Germany), retaining DHS. A subsequent application of a Microcon-YM 30 kDa column enriched both forms of eIF5A and cut off DHS protein. Protein hydrolysis was performed under nitrogen in 6 M HCl at 120°C for 24 h.

DOHH assay

DOHH substrate (i.e. eIF5A (Dhp) (Frommholz et al. 2009; Kerscher et al. 2010) was prepared as described in "Results" section. A typical assay contained: DOHH purified by nickel-chelate chromatography from *P. falciparum* NF54 strain (7.5 µg), 50 mM sodium phosphate buffer pH 7.4, 1 mM NAD^+, 1 mM DTT, and approximately 20 µg eIF5A (Dhp) in a reaction volume of 600 µl. Incubation was performed for 3 h at 37°C. The modified eIF5A protein was recovered by size exclusion chromatography and hydrolyzed in 6 N HCl at 120°C for 24 h.

Detection of hypusine in peptide hydrolysates with *ortho*-phthaldialdehyde by HPLC

The online derivatization for the primary amino acids was performed using a protocol from Agilent (Germany) with *ortho*-phthalaldehyde (OPA) in two precolumn steps. The reagent contained OPA 5 mg/ml and 2-mercaptoethanol (2-ME) 1% (v/v), in 0.4 M borate buffer pH 10.4. The amino acid sample was mixed 1:1 v/v with the OPA reagent and the reaction was performed at room temperature. The mobile phase A was a 20 mM sodium acetate solution with 0.018% triethylamine (TEA) adjusted to pH 7.2 with 1–2% acetic acid. The mobile phase B contained 20% of a 100 mM sodium acetate solution adjusted to pH 7.2 with 1–2% acetic acid and 40% acetonitrile and 40% methanol. Separation of the hydrolyzed peptides was performed after precolumn derivatization with the following gradient: the gradient was started with 100% mobile phase A, at 17 min; 60% B at 18 min; 100% B at 18.1 min flow 0.45; at 18.5 min flow 0.8; at 23.9 min flow 0.8; at 24 min 100% B and flow 0.45.

Separation was performed on an HPLC of the Agilent 1100 Series with a fluorescence detector (FDA) (excitation at 340 nm, emission 450 nm). A 200 × 2.1 mm AA column (Agilent, Germany) and a guard column were used. The flow rate was 0.45 ml/min and the injection volume was 1 µl.

Detection of deoxyhypusine and hypusine by UPLC using AccQ-Tag derivatization

The free amino acids obtained from the hydrolysate were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) reagent which reacts with both primary and secondary amino groups to stable derivatives. For derivatization, 80 µl of borate buffer was added to the hydrolyzed sample or to 10 µl of the amino acid reference solution. Next, 20 µl of reagent solution was added. The reaction mixture was mixed immediately and heated to 55°C for 10 min. After cooling down, an aliquot of the reaction mixture was used for UPLC injection. Separation followed in an AccQ-Tag ultra amino acid analysis column (2.1 mm × 100 mm) performing a UPLC amino acid analysis solution gradient. The solvent system consisted of two eluents: (A) AccQ-Taq ultra eluent A concentrate (5%, v/v) and water (95%, v/v); (B) AccQ-Tag ultra eluent B. The following gradient elution was used. First, an isocratic hold was performed at 0.1% B for 0.54 min. Then a non-linear gradient-step to 9.1% B in 5.2 min was followed by a linear step to 21.2% B in 2 min. Subsequently a linear step in 0.3 min to 59.6% B was performed. The column was flushed at 90% B and re-equilibrated at 0.1% B for 0.8 min. Separation was performed on an Acquity system (Waters) equipped with a binary solvent manager, a sample manager with column heater and a photodiode-array detector (PDA). The flow rate was 0.7 ml/min and the column temperature was kept at 55°C. The injection volume was 1 µl and the detection wavelength was set at 260 nm.

Results

Determination of covalently bound hypusine: reversed-phase HPLC after OPA derivatization versus UPLC after AccQ-Tag derivatization

In a first attempt to determine covalently bound hypusine in eIF5A, we determined DOHH activity in *Plasmodium* and human cells using purified enzyme preparations of the recombinant DHS and DOHH proteins, respectively. Peptide hydrolysates were analyzed by reversed-phase HPLC after precolumn derivatization with OPA (see "Materials and methods" section). Retention times of an amino standard solution for fluorescence detection containing 17 different amino acids (concentration 25 nmol of each amino acid/ml) and different biogenic amines (0.17 µmol/µl) were determined (Fig. 1a). The precision of retention times and

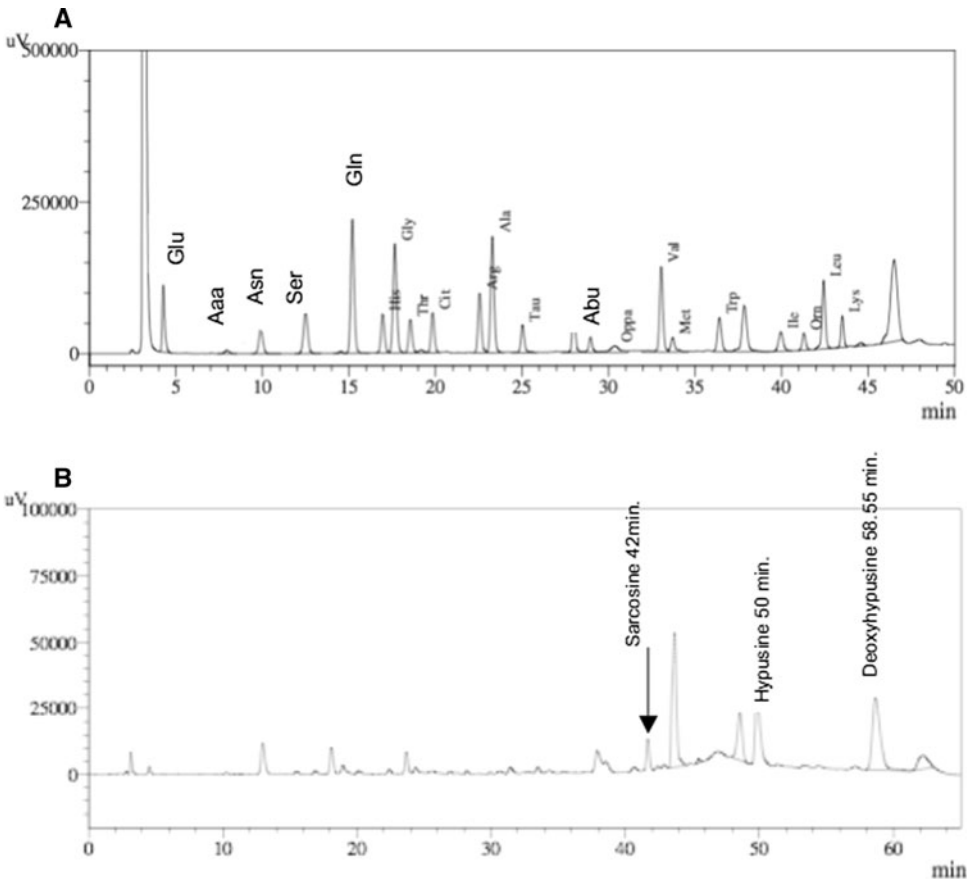

Fig. 1 **a** Chromatogram of the *ortho*-phthaldialdehyde derivatives of a standard amino acid solution containing 17 amino acids, biogenic amines and sarcosine as an internal standard after separation by reversed-phase HPLC. The amino acids were: glutamic acid (Glu) 4.27 min, L-2-aminoadipic acid (Aaa) 7.95 min, asparagine (Asn) 9.91 min, serine (Ser) 12.49 min, glutamine (Gln) 15.19 min, histidine (His) 16.95 min, glycine (Glyc) 17.65 min, threonine (Thre) 18.55 min, citrulline (Cit) 19.83 min, arginine (Arg) 22.55 min, alanine (Ala) 23.29 min, taurine (Tau) 25.03 min, tyrosine (tyr) 27.99 min, α-aminobutyric acid (Abu) 28.96 min, *ortho*-phosporylethanolamine = (*ortho*-phosphorylcolamine) (Oppa) 30.37 min, valine (Val) 33.05 min, methionine (Met) 33.71 min, tryptophane (Trp) 36.41 min, phenylalanine (Phe) 37.85 min, isoleucine (Ile) 39.96 min, ornithine (Orn) 41.29 min, leucine (Leu) 42.43 min, lysine (Lys) 44.6 min. Sarcosine was applied as a standard (1 nmol) with a retention time of 42 min (marked by the *arrow*). A standard of hypusine contained 0.829 nmol/µl. **b** A complete deoxyhypusine hydroxylase assay containing hypusine (retention time 50 min) and deoxyhypusine (retention time 48.55 min)

areas, and the limit of detection and linearity for the amino acid standard solution was 100 pmol/µl. Purified, authentic hypusine and deoxyhypusine were used as a standard which resulted in retention times of 50 min for hypusine and 58.55 min for deoxyhypusine, respectively (Fig. 1b). As an internal standard sarcosine (1 nmol) was applied which is not present in the peptide hydrolysate and has a retention time of 42.0 min (Fig. 1a, b).

To determine the specific activity of the DOHH from *Plasmodium*, we modified the eIF5A protein from *P. vivax* to eIF5A (Dhp) by human DHS which has a higher specific enzymatic activity than the parasitic enzyme (Frommholz et al. 2009). The deoxyhypusinylated eIF5A precursor protein was subsequently used in an incubation with purified DOHH from *P. falciparum* and human. Surprisingly, a specific activity for *Plasmodium* DOHH was determined which was twofold higher i.e. 7.9 µmol/mg protein in comparison to the human enzyme which had a specific activity of 3.94 µmol/mg protein (Fig. 4a, b). To our knowledge, this is the first enzyme of the hypusine pathway in *Plasmodium* which has a significant higher activity than its human counterpart. However, this analytical procedure was time-consuming and the retention times were strictly dependent on the stability of the OPA derivatives of deoxyhypusine and hypusine.

Since a rapid, precise and labour-saving method is one of the main prerequisites for a biomarker monitoring and for a HPTS, we applied UPLC analysis for the determination of deoxyhypusine (see "Materials and methods" section) and hypusine.

In a first step we analyzed chemically synthesized deoxyhypusine as a standard (2 mg/ml) after derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate (AccQ-Tag) reagent. The chromatogram in Fig. 2a shows

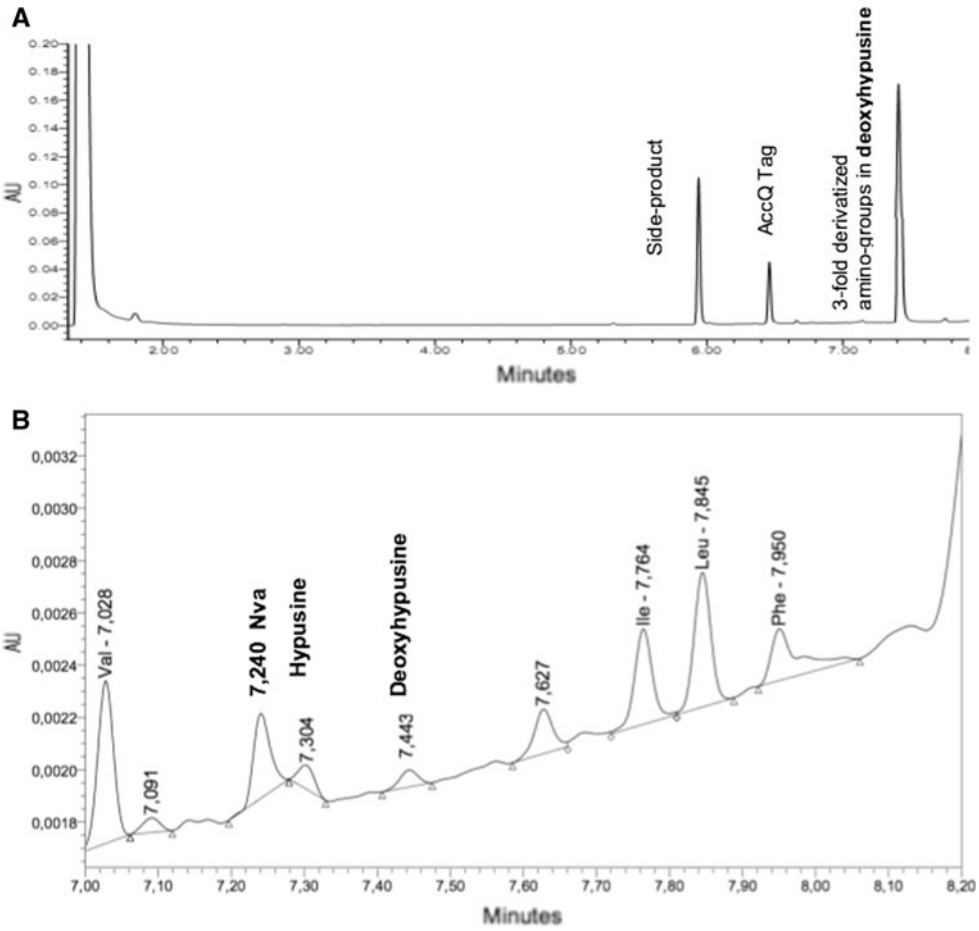

Fig. 2 UPLC-analysis of: **a** Deoxyhypusine standard (concentration 2 µg/µl) after derivatization with AccQ-Tag. Derivatization of one or two of the three amino groups resulted in a peak with a retention time of 5.939 min. However, a complete derivatization of all three amino groups with AccQ-Tag reagent leads to the main peak with a retention time of 7.764 min. **b** A complete deoxyhypusine synthase assay with recombinant, purified enzyme from *Plasmodium*. Deoxyhypusine eluted with a retention time of 7.443 min after derivatization of the three amino groups with 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate while hypusine was detected after 7.304 min. The amino acids which were present in the peptide hydrolysate were: Valine (Val), isoleucine (Ile), leucine (Leu), phenylalanine (Phe). Norvaline (Nva) was used as an internal standard in a concentration of 1 nmol

that when all three amino groups of deoxyhypusine have been derivatized, the resulting deoxyhypusine peak elutes with a retention time of 7.414 min. This peak also occurs as the main peak in the chromatogram. Alternatively, besides the derivatization of all three amino groups present in hypusine only one or two amino groups can be derivatized by AccQ-Tag. One of both possible derivatization side products is represented by the peak (side product) resulting in a retention time of 5.939 min. The third peak with a retention time of 6.458 min derives from the AccQ-Tag reagent, respectively.

Next we analyzed deoxyhypusine and hypusine in the eIF5A precursor protein present in a complete DOHH assay. Firstly, we modified the eIF5A protein from *P. vivax* to the deoxyhypusinylated form (i.e. eIF5A (Dhp) using human DHS (Frommholz et al. 2009). So eIF5A (Dhp) was isolated by two size exclusion chromatography steps (i.e. Microcon-YM 100 and 30 kDa), which cut off DHS. Subsequently, eIF5A-Dhp was incubated with purified recombinant DOHH from P. *falciparum*. Completely modified eIF5A was analyzed by peptide hydrolysis after derivatization with AccQ-Tag reagent.

Before further analysis we applied a standard UPLC amino acid analysis solution with 17 amino acids (containing 10 pmol of each amino acid in 0.1 M HCl) and norvaline (1 nmol) as an internal standard. A comparison between the OPA derivatives of the amino acids present in the amino acid standard separated by HPLC under fluorescence detection (Fig. 1a) and the AccQ-Tag derivatives separated by UPLC under photodiode-array detection (Fig. 3a) shows an improvement with respect to resolution and retention times. Deoxyhypusine was detected in the peptide hydrolysate with a retention time of 7.443 min between the amino acids norvaline (retention time 7.240 min) and isoleucine (retention time 7.764 min) (Fig. 2b). By contrast hypusine eluted with a retention time of 7.304 min. The limit of detection for deoxyhypusine and hypusine was 0.144 ng/µl.

To prove the identity of the hypusine peak in the chromatogram we spiked hypusine (green line) into the peptide hydrolysate of completely modified eIF5A. The overlay of the chromatograms from two independent peptide hydrolysates obtained from two different DOHH assays is shown in Fig. 3b (red and black lines) together

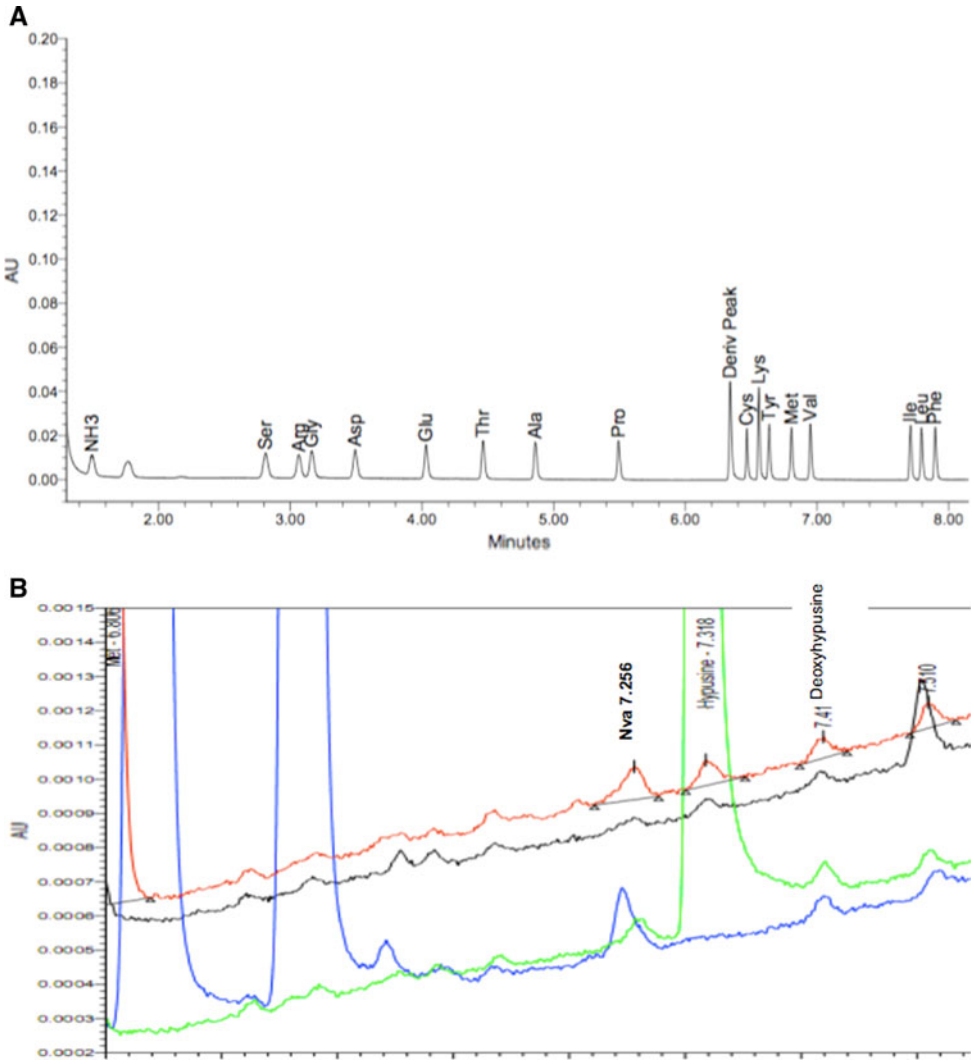

Fig. 3 **a** UPLC analysis of an amino acid standard solution with AccQ-Tag reagent: NH_3 1.494 min, serine (Ser) 2.813 min, arginine (Arg) 3.065 min, glycine (Gly) 3.163 min, asparagine (Asn) 3.493 min, glutamine (Gln) 4.031 min, threonine (Thr), alanine (Ala) 4.861 min, proline (Pro) 5.493 min, cysteine (Cys) 6.468 min, lysine (Lys) 6.559 min, tyrosine (Tyr) 6.637 min, methionine (Met) 6.806 min, valine (Val) 6.951 min, isoleucine (Ile) 7.711 min, leucine (Leu) 7.795 min, phenylalanine (Phe) 7.898 min. **b** An overlay of UPLC-chromatograms of two different peptide hydrolysates obtained from DOHH incubations (*black and red lines*) containing the internal standard norvaline (Nva) and the amino acid standard containing norvaline (color figure online)

with the internal standard norvaline. Taken together these results demonstrate that norvaline and hypusine with retention times of 7.256 and 7.318 can be clearly separated. Secondly, when we used a DHS deletion mutant from *Lycopersicon esculentum* (Solanaceae) which did not produce deoxyhypusine and hypusine (Kaiser unpublished) both amino acids were absent in the chromatogram.

The proof of principle of the UPLC assay to screen for antimalarial drugs: complexing the ferrous iron in parasitic DOHH lacks selectivity with respect to the human enzyme

Next we performed a proof of analytical principle of the UPLC assay by testing two pharmacological compounds for inhibition of hypusine biosynthesis in *Plasmodium.*

Since *Plasmodium* DOHH is known to coordinate ferrous iron by four highly conserved histidine glutamate binding sites we tested a variety of iron chelators (Clement et al. 2002). When 2,2′-dipyridyl and mimosine were applied in a concentration of 100 µM in the DOHH assay, no specific enzymatic activity was detectable for the human and the parasitic enzyme after UPLC analysis suggesting that they chelate the ferrous iron in both proteins without selectivity. Moreover, both compounds were highly toxic in vivo in a rodent malaria model (Goebel et al. 2008), and thus could not be applied as an antimalarial.

Following the iron-complexing hypothesis 4-oxo-piperidine-carboxylates were applied with a potential of chelating the metal either via the enolizable β-ketoester moiety or via the three nitrogens, in position 1 and in the pyridines (Goebel et al. 2008). EHW 437 (4-[2,6-dichlorobenzyloxyimino)methyl]-1-(3-phenylpropyl) piperidinium hydrochloride (Fig. 4) represents an oxime ether of the 4-oxo-piperidine-carboxylate while JK8-2 is a nitrophenylderivative (Goebel et al. 2008) (Fig. 4). Both EHW 437 and JK8-2 compounds were recently tested for their inhibitory properties in in vitro cultures from *P. falciparum* and found to be more

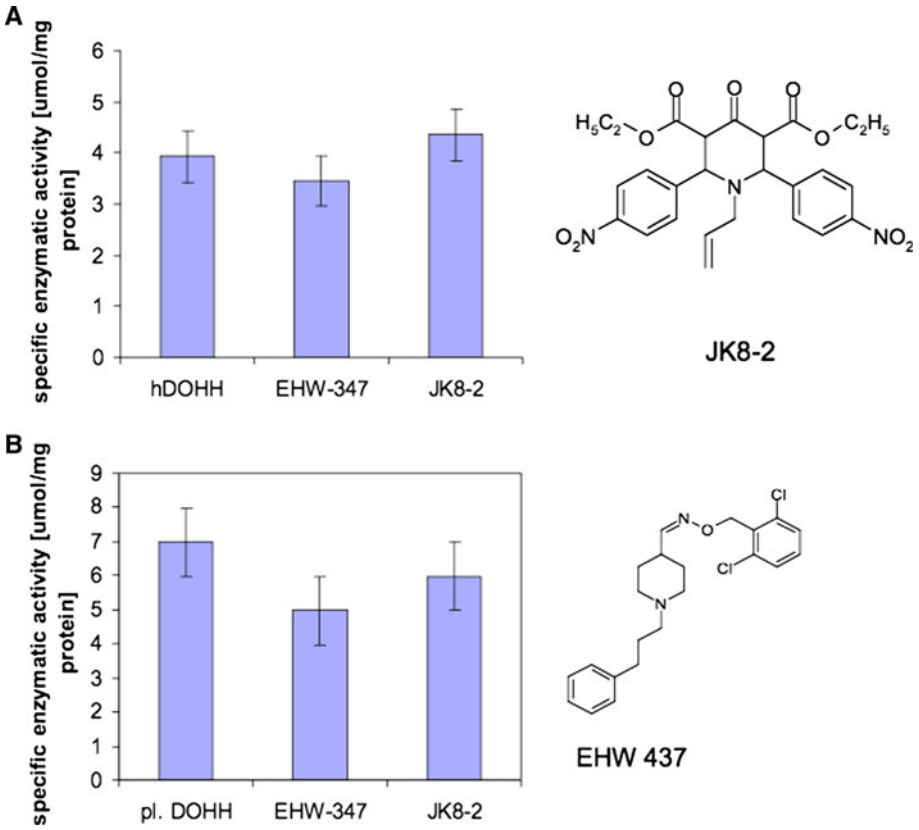

Fig. 4 Specific enzymatic activity of deoxyhypusine hydroxylase after inhibition with 8 μM JK8-2 and 8 μM EHW 437. **a** Inhibition of the purified human enzyme. **b** Inhibition of the purified *P. falciparum* deoxyhypusine hydroxylase. *The left column* represents the specific enzymatic activity of the human or the plasmodial enzyme without inhibitor. Standard *error bars* represent the mean of three independent experiments

active than mimosine with EC_{50} values of 8.29 μM for EHW 437 and 11.03 μM for JK8-2, respectively (Goebel et al. 2008). These results prompted us to determine hypusine formation in a DOHH assay using 100 μM of each of both inhibitors and purified DOHH preparations from *Plasmodium* and human. As shown in Fig. 4 the specific enzymatic activities of DOHH did not significantly change, independently whether one of both compounds was present or absent in the assay. These results suggest that both compounds do not chelate the ferrous iron present in human or plasmodial DOHH.

Discussion

Here, we demonstrate that hypusine and its precursor deoxyhypusine can be rapidly detected and quantified by UPLC with retention times of 7.443 min for deoxyhypusine and 7.304 min for hypusine, respectively.

Derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate (AccQ-Taq) leads to stable derivatives of all three amino groups present in hypusine resulting in a main peak of 7.304 min. This analytical method can replace previous HPLC methods with retention times detecting the *ortho*-phthaldialdehyde derivatives of deoxyhypusine at 17.4 min and hypusine at 17.4 min (Beppu et al. 1996; Beninati et al. 1990). Moreover, reproduction of these experiments showed that a gradient performed with mobile phase A consisting of 20 mM sodium acetate and mobile phase B consisting of 40% methanol and 40% acetonitrile prolonged retention times of deoxyhypusine to 58.55 min and hypusine to 50 min (Fig. 1b). The *ortho*-phthaldialdehyde derivatives lacked reproducibility because of their instability. A third alternative method analyzed the hydrolyzed amino acids after derivatization with 4-dimethylaminoazobenzene-4-sulphonylchloride (dabsylation) by reversed-phase high-performance chromatography in a precolumn step (Bartig and Klink 1992). However, DABS-hypusine shows a retention time of 16 min in the chromatogram which is not applicable for a HPTS-assay. The limit of detection was in the fmol range (Bartig and Klink 1992). By contrast, the novel assay performed by UPLC showed a limit of detection of 0.144 ng/μl.

Standardization curves for hypusine and deoxyhypusine in amounts of 5–200 pmol were employed. Regression analysis indicated no significant deviation from linearity with these amounts of either amino acid (data not shown). Retention time reproducibility was found to be excellent. An average standard deviation of ±4% for either hypusine or deoxyhypusine was determined. In the future we intend to determine both amino acids from biological samples to evaluate the potential of eIF5A as a biomarker. Experiments are currently underway.

Two new interesting results with respect to hypusine biosynthesis were obtained from the quantification

performed by the UPLC assay. (i) DOHH from *Plasmodium* has a twofold higher specific activity than its human counterpart. One reason might be that the DOHH from *Plasmodium* consists of five rather than four E-Z type heat like repeats present in the human counterpart (Zhao et al. 2004). The plasmodial E-Z heat-like repeats show significant homology to those present in phycocyanin lyase subunits (Zhao et al. 2004) of cyanobacteria. These E-Z type heat like repeat domains might contribute to an improved binding of deoxyhypusinylated eIF5A (Dhp) and therefore lead to an increase of the specific enzymatic activity. (ii) The purified recombinant human DOHH enzyme was a mixture of active holoenzyme containing 2 mol of iron/mol of enzyme and the metal-free apoenzyme (Kim et al. 2007). The iron of human DOHH was coordinated by strictly conserved His–Glu residues which were also present in parasitic DOHH (Clement et al. 2002). Following the iron-complexing strategy our results showed that 2,2′-dipyridyl and mimosine were potent inhibitors of both enzymes and therefore lacking selectivity towards the parasitic DOHH. In an attempt to screen for selective iron chelators of parasitic DOHH we tested inhibition of hypusine with EHW 437 and JK8-2 by UPLC analysis. Although both compounds have been highly active in vitro and in vivo (Saeftel et al. 2006; Goebel et al. 2008) they did not inhibit the purified DOHH (Fig. 4). Thus it seems likely that both inhibitors complex different metal ions of enzymes being involved in proliferation.

In between hypusine has been determined in biological samples i.e. in erythrocyte infected malaria parasites to evaluate the pharmacodynamic potential of this posttranslational application as a biomarker in antimalarial therapy. A significant increase of hypusine from early to late developmental stages of the parasite was monitored which paralleled the occurrence of the triamine spermidine during infection. Remarkably, uninfected erythrocytes contained hypusine i.e. 2.5 pmol hypusine per 10^6 erythrocytes although they lack nuclei with the genetic information for the biosynthetic enzymes. This finding might result from the surroundings caused by a distinct transport system. After 48 h of parasitic development 40 pmol hypusine per 10^6 infected erythrocytes was formed.

The rapid and robust detection of hypusine by UPLC will be a challenge to monitor hypusine formation in different developmental stages of the parasite to refine a patient's care with respect to diagnosis of severe illness and therapy.

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Conflict of interest The authors declare that they have no conflict of interest.

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