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Determination of the trypanocidal drug melarsoprol and its conversion products in biological fluids with HPLC–ICPMS/ESMS

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ABSTRACT

Although melarsoprol, an organoarsenic compound, is widely used for the treatment of trypanosomiasis (human African sleeping sickness), very little is known about its fate in the human body, its active metabolites passing the blood–brain barrier and the mode of action. Previous pharmacological studies based on the determination of melarsoprol by HPLC–UV or by a bioassay method produced different results. We report a HPLC–ICPMS method suitable for determining melarsoprol and its metabolites in biological fluids. The arsenic selective capability of the method allowed the quantitative measurement of melarsoprol and two arsenic-containing conversion products produced when melarsoprol was incubated with human serum and blood. The major product was identified as melarsen [4-[(4,6-diamino-1,3,5-triazin-2-yl)amino]phenyl]arsonic acid by HPLC/electrospray MS, and by accurate mass measurements. Investigations about the stability of melarsoprol in serum showed that within 30 h about 10% of melarsoprol is converted to melarsen. In blood, however, most of the melarsoprol was bound to proteins and only 1% was converted to melarsen after 30 hours. The limit of detection for melarsoprol and its conversion products were in the range of 1 μg As L^{-1} (13 nmol As L^{-1}) based on signal to noise ratio of 3 with a 10 μL injection volume allowing direct determination of the compounds in blood and serum (after protein precipitation) at therapeutically realistic concentrations.

1. Introduction

Human African trypanosomiasis, or sleeping sickness, is one of the most complex of all endemic tropical diseases. Parasites transmitted by the bite of the tsetse fly initially circulate in the bloodstream and lymphatics, for weeks to years, before crossing the blood–brain barrier to infect the central nervous system (CNS). The disease is widely prevalent in sub-Saharan Africa with tens of thousands of people infected and over 60 million people at risk [1].

Melarsoprol, a trivalent organic arsenical (Fig. 1), was introduced as a treatment against CNS trypanosomiasis in 1949 [2]. The drug is administered intravenously as a 3.6% solution in propylene glycol. Although effective against the parasite, the treatment elicits severe adverse reactions; in particular encephalopathic syndromes occur in 2–10% of patients, leading to death in 50–70% of these [3]. Despite its high toxicity to humans, melarsoprol remained the drug of choice for CNS trypanosomiasis until 2000. The search for an effective and less toxic drug is continuing. One of the drugs tested was eflornithine, an inhibitor of ornithine decarboxylase [4], but it was shown to be ineffective against *Trypanosoma brucei rhodesiense*, and its short duration of action and low efficacy caused logistic problems [5]. Today the nifurtimox–eflornithine combination treatment (NECT) is the preferred treatment for trypanosomiasis [6].

In parallel with the search for a safer drug for treating CNS trypanosomiasis, there are ongoing investigations into safer and more effective ways to administer melarsoprol. Integral to these pharmacological studies is an understanding of the mode of action of melarsoprol and its human metabolism, and hence analytical methods have been developed to determine melarsoprol and its possible metabolites in biological fluids. In general, two types of methods have been employed. Melarsoprol can be determined directly by using reversed-phase HPLC with UV detection [7]; or melarsoprol concentrations can be measured indirectly with a bioassay that determines the parasitical activity in body fluids [8,9]. Both methods, however, have limitations in terms of selectivity, so neither of the methods is able to reliably follow the formation of conversion products from melarsoprol [10].

Further analytical problems appear when one compares the pharmacological data obtained from the different analytical methods. Large discrepancies in the concentrations of melarsoprol in

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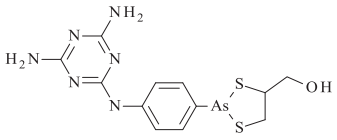
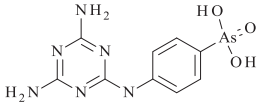


"Melarsoprol"

[2-[4-[(4,6-diamino-1,3,5-triazin-2-yl)amino]phenyl]-1,3,2-dithiarsolan-4-yl]methanol

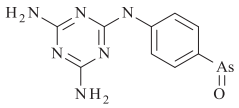
($C_{12}H_{15}AsN_6OS_2$; Molecular Weight : 398.34)



"Melarsen"

[4-[(4,6-diamino-1,3,5-triazin-2-yl)amino]phenyl]arsonic acid

($C_9H_{11}AsN_6O_3$ Molecular Weight : 326.14)



"Melarsen oxide"

N4-(4-arsorosophenyl)-1,3,5-triazine-2,4,6-triamine

($C_9H_9AsN_6O$ Molecular Weight : 292.13)

Fig. 1. Structures of compounds mentioned in this work.

biological fluids have been observed depending on whether the measurements were made directly by HPLC–UV, or indirectly by bioassay, which usually gives much higher values [10,11]. This discrepancy translates into widely differing estimates of the half-life of melarsoprol in the body, ranging from less than one hour for studies based on HPLC–UV results to up to 35 h for studies that employed bioassay for the measurements. Trypanocidal activity (in vitro studies) has also been reported for several derivatives of melarsoprol, and the activity depended on the arsenic concentration and also on its oxidation state, where trivalent arsenic species showed higher trypanocidal activity [10,11]. Keiser et al. [11] investigated the metabolites of melarsoprol in blood, serum and cerebrospinal fluid; they found that a significant portion of melarsoprol was bound to proteins and they also identified melarsen oxide as one metabolite of melarsoprol in serum and blood. Identification of this compound was done by HPLC–UV based on retention time matching. Melarsen oxide was not detected in cerebrospinal fluid at quantification limits of 50 ng · compound · L^{-1}. When the authors compared the time courses of the concentrations of melarsoprol and melarsen oxide in serum after injection of melarsoprol with the total concentration of arsenic (determined by GFAAS) a huge discrepancy was observed. In samples taken 24 h after the injection, neither melarsoprol nor melarsen oxide could be determined by HPLC, but trypanocidal activity and arsenic could still be detected [11]. Collectively, these findings indicate that an unknown metabolite of melarsoprol, not identified by HPLC–UV, is responsible, at least in part, for the trypanocidal activity observed in the bioassay tests. Unknown conversion products of melarsoprol were also proposed to be responsible for the adverse reactions observed when melarsoprol was used in the treatment of human African sleeping sickness [3].

The current discrepancies between pharmacological studies on melarsoprol based on measurements by HPLC–UV or bioassays highlight the need for an improved analytical method capable of measuring melarsoprol and its possible conversion products in biological fluids. The types of biological fluid should include blood, serum and ultimately cerebral interstitial fluid since this is likely to be the most effective site to combat the parasite [12]. Analysis of these biological fluids also demands a sensitive method particularly in the case of cerebral interstitial fluid where melarsoprol levels are 50–100 times lower than those in plasma [8,12]. HPLC/ICPMS offers the advantages of element selectivity combined with low limits of detection [13,14] which could help to answer the pharmacokinetic discrepancies mentioned above. We report here an analytical approach based on cation-exchange HPLC coupled to ICPMS as the element selective detector for determining melarsoprol and its arsenic-containing conversion products.

2. Experimental

2.1. Standards and reagents

Water used throughout this study was obtained from a Milli-Q system (18.2 MΩ cm; Millipore GmbH Vienna Austria). The following commercial products were used: pyridine (analytical grade p.a.), formic acid (96%, analytical grade p.a.), and ethanol (LiChrosolv) from Merck (Buchs, Switzerland); hydrogen peroxide (30% p.a.), 2-propanol and propylene glycol were obtained from Carl Roth GmbH (Karlsruhe, Germany). Methanol (chromasolv) and acetic acid (99.8%) used for the high resolution ESMS measurements were obtained from Sigma-Aldrich (Vienna, Austria). Melarsoprol (trade name Arsobal) was manufactured by Aventis, France and obtained through the Centers for Disease Control and Prevention (Atlanta, Georgia, USA). The drug is provided in an ampoule containing 180 mg melarsoprol in 5 mL propylene glycol (3.6% m/v). Arsenocholine (used for external calibration in HPLC–ICPMS measurements) was synthesized in-house.

2.2. Serum and blood samples

Blood (ca 16 mL) was obtained from one volunteer (male, 46 years old) in 8 mL vacuette®heparin tubes (Greiner bio-one, Frickenhausen, Germany). Serum of the same volunteer was obtained in 8 mL vacuette® serum separator tubes (Greiner bio-one, Frickenhausen, Germany). Serum samples were obtained after centrifugation at 1500*g* for 10 min at 4 °C. The blood and serum samples were stored at 4 °C (2 days) until analysis.

2.3. Instrumentation

ICPMS measurements were performed with an Agilent 7500ce, and HPLC was carried out with an Agilent 1100 series instrument (Agilent Technologies, Waldbronn, Germany). The ICPMS was equipped with a Burgener Ari Mist HP nebulizer (Burgener Research Inc, Mississauga, Canada) and a ESI PC3 peltier-cooled cyclonic spray chamber (Elemental Scientific Omaha, Nebraska, USA). The HPLC was equipped with a binary pump, a vacuum degasser, column oven, an autosampler with a variable 100 μL injection loop and a fraction collector. The HPLC was connected to the ICPMS with 0.125 mm internal diameter PEEK (polyetheretherketone) tubing (Upchurch Scientific, Oak Harbour, USA).

For ESMS measurements, an Agilent LCMSD single quadrupole mass spectrometer of the SL type was used. The mass spectrometer was equipped with an atmospheric pressure ionization (API) source employing pneumatically assisted electrospray nebulization with nitrogen as the nebulizer gas. The mass spectrometer was tuned automatically with the calibrant solution for API provided by Agilent. The system was operated in the positive mode. As the nitrogen source, a Parker NitroFlow®Lab (Parker Filtration & Separation B.V., AG-Etten-Leur, The Netherlands) nitrogen generator was used. A drying gas temperature of 350 °C, and a drying gas flow of 11 L min^{-1} were used. The nebulizer pressure was adjusted to a back pressure of 2.1 bar, and the capillary voltage was set to 3.5 kV. In positive scan mode the mass range was set from *m/z* 100 to *m/z* 500; fragmentor voltage was 150 V. In the SIM mode the fragmentor

voltages for the determination of pseudomolecular ions $[MH]^+$ were set at m/z 327 (150 V) and 399 (150 V).

2.3.1. HR-ESMS

After the HPLC, the fraction containing the highest concentration of the conversion product was diluted in 80% aqueous methanol containing 0.1% acetic acid to give a solution containing approx. 1 μg As mL^{-1}. The measurements were carried out on a LTQ orbitrap system equipped with an ESI source (Thermo Fisher Scientific). The samples were introduced via the syringe pump using a flow rate of 5 μL min^{-1} into the mass spectrometer operated under the following conditions: positive mode, spray voltage: 4 kV, capillary Temp: 275 °C, gas flow rate: 10 arbitrary units. The spectra were acquired in full scan mode and MS/MS experiments were carried out with various fragmentor voltages (0–40 V).

2.4. Sample preparation

2.4.1. Treatment of melarsoprol with H_2O_2

Hydrogen peroxide was added in a concentration of 0.03% to solutions of melarsoprol (70 μmol L^{-1}) in propylene glycol and repetitive HPLC–ICPMS/ESMS measurements were performed over 45 min. The experiment was performed to identify possible conversion products of melarsoprol by HPLC–ICPMS/ESMS since the original drug is a trivalent arsenic species which should be susceptible to oxidation.

2.4.2. Incubation experiments

The applicability of the HPLC–ICPMS method was tested with blood and serum samples, from one volunteer, that had been spiked with melarsoprol and incubated at 37 °C for 30 h. The amount of melarsoprol added was calculated from the typical dose administered (2.2 mg melarsoprol per kg body weight) for the usual treatment of trypanosomiasis. With a body weight of 80 kg and a total blood volume of 5 L, the concentration of melarsoprol in blood is around 90 μmol L^{-1} which corresponds to 6.8 mg As L^{-1}. About 10 g of whole blood and 5 g of serum were weighed with a precision of 0.1 mg into 50 mL polypropylene tubes. Melarsoprol in propylene glycol was added to the samples to give a nominal concentration of 90 μmol L^{-1}. Samples were incubated in a GFL-1083 shaking water bath (Gesellschaft für Labortechnik, Burkwedel, Germany) at 37 °C for 36 h. After time periods of about 2 h, aliquots of whole blood (200 μL) and serum (100 μL) were transferred to micro-centrifuge tubes (1.5 mL, BRAND) containing the same volume of cold acetonitrile. The suspension was centrifuged at 8900 g for 15 min in a microcentrifuge. The supernatant was directly used for HPLC–ICPMS measurements after dilution 1+19 with water. After decanting the supernatant the pellet was washed with 1 mL H_2O-acetontrile (1+1) and again centrifuged at 8900g for 5 min.

2.4.3. Determination of total arsenic

Each liquid sample (blood, serum) was analyzed for total arsenic content in duplicate; the pellets after acetonitrile precipitation were analyzed for total arsenic as single measurements. Analysis was performed in the following manner: a portion of the liquid samples (about 250 mg weighed with a precision of 0.1 mg) was weighed directly into 12 mL quartz tubes, and HNO_3 (2 mL) and H_2O (2 mL) were added. The pellet was suspended in HNO_3 (2×1 mL), transferred to 12 mL quartz tubes and H_2O (2 mL) was added. The tubes were transferred to a Teflon® rack of the Ultraclave microwave system (MLS GmbH, Leutkirch, Germany) and covered with Teflon® caps. After closing the system, an argon pressure of 4×10^6 Pa was applied and the mixture was heated to 250 °C for 30 min before being allowed to cool to room temperature. After mineralization, the samples were transferred to 15 mL polypropylene tubes (Greiner, Bio-one, Frickenhausen, Germany) and diluted with water to 9 mL (based on mass). Finally 1 mL of a solution containing 50% methanol (to enhance the arsenic response) and 100 μg L^{-1} each of Ge and In as internal standards was added to all digested samples giving a final concentration of 5% methanol and 10 μg L^{-1} of Ge and In. All standards for total arsenic determinations were prepared with 20% nitric acid and 5% methanol for matrix matching with the digested samples. The arsenic concentrations in the digests were determined by ICPMS using helium as collision cell gas for removing polyatomic interferences from argon chloride ($^{40}Ar^{35}Cl$ on ^{75}As). The accuracy of the method for the determination of total As after microwave-assisted acid digestion was validated against the certified reference material ERM BC-211 (rice) which has a certified arsenic content of 260 ± 13 μg As/kg; we obtained 258 ± 5 μg As kg^{-1} ($n=3$).

2.4.4. On line HPLC/ICPMS-ESMS analyses

Separation of the arsenic species was performed with cation-exchange HPLC by using a Zorbax SCX-300 column (4.1 × 150 mm^2 5 μm particle size). The mobile phase was a mixture of 20 mM pyridine buffer pH 3.5 (adjusted with aqueous formic acid) and 2-propanol (9+1, v/v); flow rate was 1.0 mL min^{-1}, and injection volume was 10 μL. The column effluent was split whereby 10% was directed to the ICPMS and 90% to the ESMS using a passive splitter (ASI Analytical Scientific Instruments, Richmond, USA) and introducing a sheath flow of 1.0 mL min^{-1} (0.1% formic acid). Electrospray MS data were obtained in positive mode using scan (m/z 100–m/z 500) and selected ion monitoring. The ICPMS signals at m/z 75 (^{75}As, $^{40}Ar^{35}Cl$) and m/z 77 ($^{40}Ar^{37}Cl$, to ascertain possible chloride interference on m/z 75), were monitored using a dwell time of 300 ms. An optional gas (1% CO_2 in argon) was introduced through a T-piece connecting the spray chamber and the torch to enhance the arsenic response, as first reported for selenium [14]. The data evaluation was carried out with chromatographic software G1824C Version C.01.00 (Agilent, Waldbronn, Germany). The quantification of melarsoprol and its conversion products was done by HPLC–ICPMS against external calibration with arsenocholine based on peak areas.

For speciation analysis of melarsoprol and its conversion products no certified reference materials are available. Column recoveries (ratio of sum of species divided by total As in samples applied to the column determined by flow injection experiments) were determined for all HPLC/ICPMS measurements with 104 ± 6% ($n=33$). For incubation experiments, mass balances (soluble As+protein-pellet bound As divided by total As in blood or serum) were 98 ± 3% (n=9) for serum and 110 ± 3% (n=9) for blood samples.

3. Results and discussion

3.1. HPLC–ICPMS and HPLC–ESMS of melarsoprol and its conversion products

The HPLC–UV methods for melarsoprol developed by Ericsson et al. [6] and Keiser et al. [11] are based on a reversed phase chromatographic separation, using a mobile phase consisting of up to 20% acetonitrile. Although these conditions were well-suited to the retention of melarsoprol, the use of 20% acetonitrile was not optimal for our ICPMS detection system. Delivering mobile phases high in organic solvents to the plasma of the ICPMS necessitates changes to the instrumental set-up to minimize carbon depositions on the cones [15]. Although gradient elution reversed phase HPLC–ICPMS has already been used to elute analytes with quite different polarities [16,17], we tried to avoid these complications by investigated different chromatographic systems utilizing mobile phases with a lower organic solvent content under isocratic conditions.

The amino groups of melarsoprol (Fig. 1) can be readily protonated [5] resulting in a positively charged molecule that could be amenable to cation-exchange HPLC. Therefore cation-exchange chromatography on a silica-based column was used to minimize non-polar interactions and allow elution of melarsoprol with predominantly aqueous mobile phases. Good retention and peak shape was obtained by using a Zorbax SCX-300 cation-exchange column with a mobile phase consisting of 20 mM pyridine pH 3.5 with 10% 2-propanol (v/v) (Fig. 2).The use of only 10% 2-propanol precluded the requirement for changes to the ICPMS to keep the plasma stable and to avoid carbon depositions.

Analysis of melarsoprol under these cation-exchange HPLC–ICPMS conditions produced one major arsenic peak (RT 6.8 min) together with two smaller peaks eluting at RT 3.0 min and at RT 4.5 min (Fig. 2). The small signal at the void volume (RT 1.9 min) is associated with the injection of propylene glycol. These peaks at RT 3.0 and 4.5 min are likely conversion products, notwithstanding the fact that the melarsoprol was measured immediately after the melarsoprol/propylene glycol solution had been diluted. We presumed these degradation products were oxidation products, based on the following two observations. First, melarsoprol is a trivalent arsenic species which can – like other As(III) compounds – easily be oxidized by oxygen. Second, the relative amounts of the degradation products increased with decreasing initial concentration of melarsoprol, suggesting that the transformation was effected by oxygen in the solvents.

pH optimization studies from pH 2.7 to pH 5.0 (Fig. S1) showed that with increasing pH the retention times decreased for all species, although the product at RT 3.0 min (Fig. 2) was most strongly affected. Optimal separation of melarsoprol and its conversion products could be achieved with a mobile phase comprising 20 mmol L^{-1} pyridine at pH 3.5 with formic acid containing 10% (v/v) 2-propanol.

By using HPLC–ESMS in combination with HPLC–ICPMS, we showed that melarsoprol diluted with propylene glycol (molecular mass 398; $[M+H]^+$, *m/z* 399) was converted to a product with a molecular mass of 326 ($[M+H]^+$, *m/z* 327) showing a retention time of 3 min. This is consistent with the oxidative cleavage of the arsenic thiol to give the corresponding arsonic acid, namely [4-[(4,6-diamino-1,3,5-triazin-2-yl)amino]phenyl]arsonic acid ("melarsen" Fig. 1). The assignment was confirmed by HR ESMS after collecting the fraction containing the peak of nominal mass 326. The obtained $[M+H]^+$ mass was 327.0178 the calculated mass for $C_9H_{12}N_6O_3As$ is 327.0181 (Δm −1.0 ppm). Furthermore, MS/MS experiments produced results entirely consistent with this assignment (Fig. S2 Supporting information). The MS/MS spectrum of *m/z* 327 contained one main peak at *m/z* 309.0073 corresponding to the loss of water. Fragmentation of this ion lead to a second loss of water (*m/z* 290.9969), but also to re-arrangements where either the triazine ring is lost (*m/z* 181.9581) or the oxygen binds to the ring system replacing arsenic (*m/z* 217.0833 and *m/z* 218.0909).

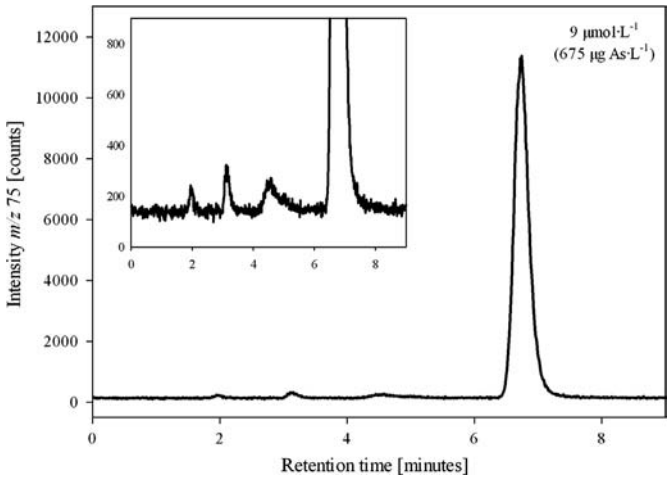


Fig. 2. HPLC–ICPMS chromatogram of melarsoprol in propylene glycol solution. Zorbax 300-SCX silica based cation-exchange column 4.1 × 150 mm, 5 µm particle size; mobile phase: 20 mM pyridine, adjusted to pH 3.5 with acetic acid; 10% 2-propanol (v/v); Flow rate: 1 mL min^{-1}; 30 °C; post column split between ESMS (90%) and ICPMS (10%); injection volume 10 µL.injection volume 10 µL.

3.2. Induced conversion of melarsoprol by H_2O_2

The possibility of additional conversion products was investigated through oxidation experiments. After addition of H_2O_2 to melarsoprol dissolved in propylene glycol, a peak at a retention time of 6 min appeared in addition to melarsoprol with a retention time of 6.8 min and melarsen at 3 min (Fig. 3). Simultaneous ESMS measurements showed a dominant mass fragment $[M+H]^+$ of *m/z* 415 corresponding to the pentavalent As oxidation product of melarsoprol. Again this peak was collected and subjected to high resolution mass spectrometry. By HR ESMS measurements the obtained mass $[M+H]^+$ was 414.99869 indicating an elemental composition of $C_{12}H_{16}N_6$ O_2AsS_2 which has a calculated mass of 414.99866 (Δm 0.06 ppm). Keiser et al. [11] identified melarsen oxide as one metabolite in serum and blood. Our studies confirmed these findings by HPLC–ESMS. Since this compound contains the arsenic still in its trivalent form further conversion products were investigated in our studies, where As is oxidized to its more stable pentavalent oxidation state. The results from these experiments are further confirmed by a recent study performed by Baumann et al. [18]. When applying potentials of > 1000 mV to solutions of melarsoprol conversion products such as the oxidized form of melarsoprol $[M+H]^+$ 415, melarsen oxide $[M+H]^+$ 293 and melarsen $[M+H]^+$ 327 were detected by ESI-ToF/MS [18].

A time course series of measurements of the melarsoprol solution containing 0.03% H_2O_2 showed that within 45 min melarsoprol, which accounted for 97% of the total As present in the original sample, was mainly converted to melarsen with a *m/z* 327 (53%), while 15% of the total As is present as the oxidized form of melarsoprol (*m/z* 415) and 30% remained as the unchanged compound (Fig. 4).

Results from incubation experiments with serum showed that about 7% of the added melarsoprol was immediately bound to proteins (Fig. 5A). The fraction of protein-bound As increased with incubation time and reached a steady state after 6–8 h, when about 18–20% was found in the pellet containing the precipitated proteins. The reason for this apparent saturation of protein-bound As is not clear, since proteins are in excess and therefore also binding sites for the trivalent As compound should be available. Conversion products of melarsoprol were detected at low levels ($\leq$ 2% of total As) immediately after addition of the drug to serum. With incubation time, the mass fraction of melarsen increases and reaches a steady state after 6–8 h with 8–12% of the total As present in the form of this species. There was a concomitant decrease in the concentration of soluble melarsoprol, which accounted for about 70% of the total As after 8 h of incubation.

The concentration of the unknown species (Fig. 2, RT 4.5 min) increased during the first 8 h of incubation reaching a maximum of 6% of the total As but then decreased again to < 2% of the total As after 30 h. Simultaneous ESMS measurements showed a mass fragment $[M+H]^+$ of *m/z* 293 corresponding to melarsen oxide (Fig. 1). Unfortunately, concentrations in blood and serum were too low to confirm this assignment by HR ESMS measurements. The time course data from the incubation experiments indicate that the purported melarsen oxide is a precursor for melarsen, since its

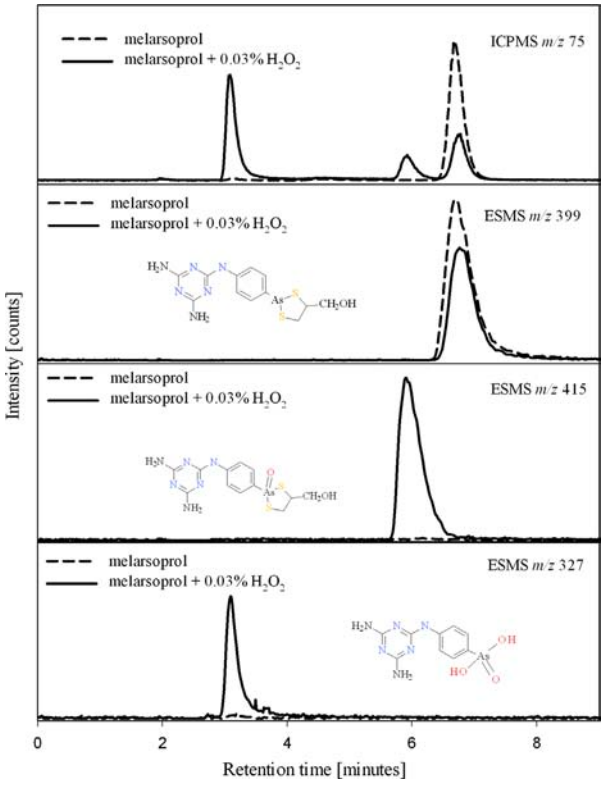


Fig. 3. HPLC–ICPMS chromatograms of melarsoprol in propylene glycol solution. Zorbax 300-SCX silica based cation-exchange column 4.1 × 150 mm, 5 μm particle size; mobile phase: 20 mM pyridine, adjusted to pH 3.5 with formic acid; 10% 2-propanol (v/v); Flow rate: 1 mL min^{-1}; 30 °C; post column split between ESMS (80%) and ICPMS (20%); injection volume 10 μL.

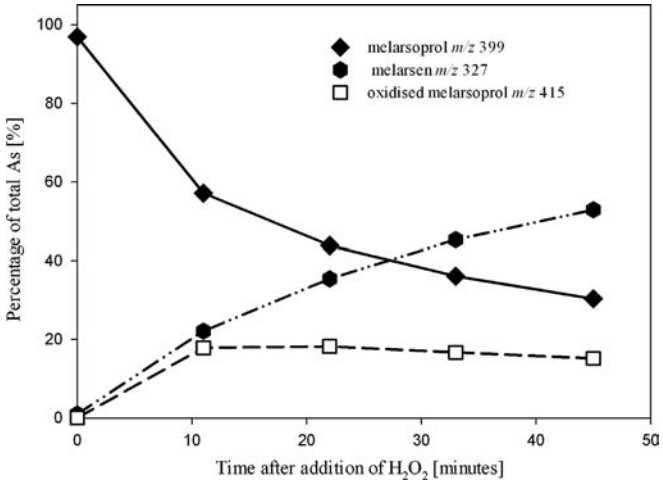


Fig. 4. Oxidation experiments of melarsoprol (70 μmol L^{-1}) with 0.03% H_2O_2. A small amount of melarsen oxide remained essentially unchanged at 1% of total As (data not shown in the graph). Chromatographic conditions: Zorbax 300-SCX silica based cation-exchange column 4.1 × 150 mm, 5 μm particle size; mobile phase: 20 mM pyridine, adjusted to pH 3.5 with formic acid; 10% 2-propanol (v/v); Flow rate: 1 mL min^{-1}; 30 °C; post column split between ESMS (80%) and ICPMS (20%); injection volume 10 μL.

concentration in serum peaks at 6% of the total As after 8–11 h and then decreases to about 2% of the total As at 30 h, while the concentration of melarsen increases between 11–30 h from 7% to 12% of the total As. Since melarsen is a trivalent arsenic species, it is likely to be oxidized to melarsen by dissolved oxygen.

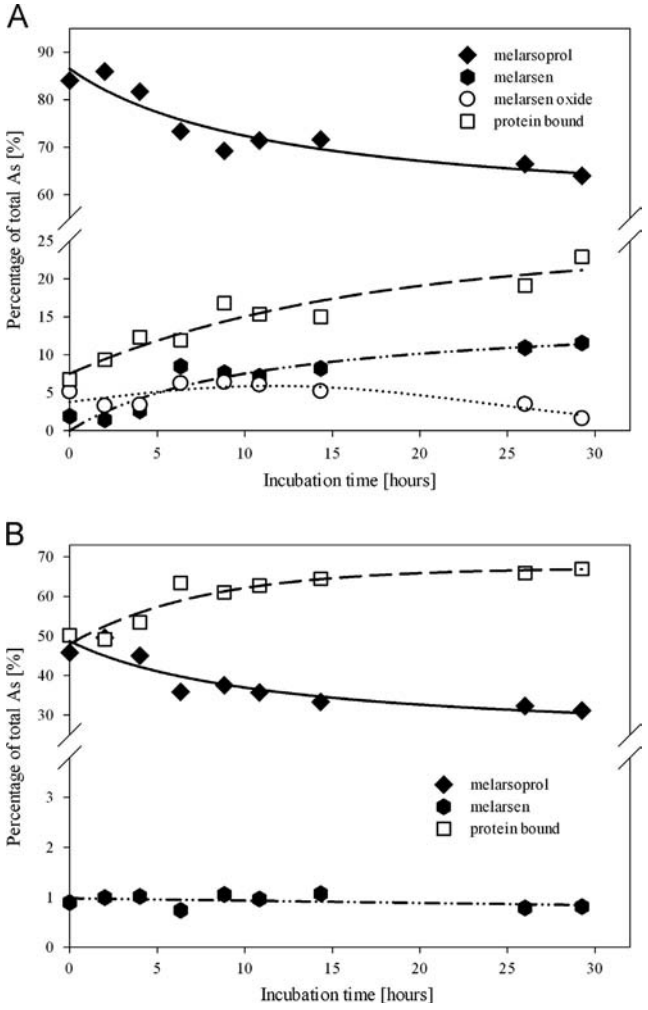


Fig. 5. Incubation experiments: Serum (A) and blood (B) spiked with melarsoprol at 90 μmol L^{-1} and incubated at 37 °C for 30 hours. Quantitative results obtained by HPLC/ICPMS (melarsoprol, melarsen and melarsen oxide) after protein precipitation and after microwave assisted acid digestion (total As, protein bound As). Mass balances 98 ± 3% for serum and 110 ± 3% for blood.

In blood melarsoprol behaved quite differently. The fraction of protein-bound As is significantly higher compared to serum with about 50% of the total As being immediately bound after adding the drug (Fig. 5B). Similar to the situation with serum, the fraction of protein-bound As in blood reached a steady state after 6–8 h, while the concentration of dissolved melarsoprol in blood decreased from 50% to 35% within 30 h of incubation. Conversion products like melarsen or melarsen oxide were present only in trace amounts accounting for about 1% of the total As. In contrast to serum, the fraction of melarsen remained constant over time constituting about 1% of the total As concentration.

It has to be pointed out at this stage, that previous pharmacological studies have been performed with only limited knowledge of which As species were present and at what concentrations. The intact drug has never been identified in the brain after passing the blood–brain barrier; the data only show a correlation between trypanocidal function in cerebrospinal fluid and arsenic concentrations in blood and serum [11]. Our studies show, that even without any human metabolic action, several conversion products of the drug can already be observed in blood and serum.

The analytical method presented here is sensitive (LOD 13 nmol L^{-1}) and also arsenic selective. The HPLC method based

on cation exchange chromatography allowed both the identification of melarsoprol and its conversion products (with ESMS), and their selective quantification at low concentrations (ICPMS). The ESMS data from incubation experiments showed two main conversion products of melarsoprol: melarsen oxide, a trivalent arsenic species previously identified as a metabolite of melarsoprol [11], and melarsen which accounted for the highest mass fractions of all conversion products in serum. Our studied confirmed the previously observed short lifetime of melsarsen oxide which is a precursor for the formation of melarsen. The ultimate question to answer will be to identify the arsenic species passing the blood–brain barrier. The methods used until now were not sensitive and selective enough to identify the arsenic species in CSF or – even more important – in the interstitial fluid directly in the brain. Therefore the method described here based on HPLC–ICPMS could be a tool to find out the trypanocidal active arsenic species passing the blood–brain barrier but maybe also to identify the compound responsible for the adverse effects observed during treatments of human African trypanosomiasis with melarsoprol. Its application to pharmacological studies will better define the toxic or active metabolites.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.07.066.

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