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Determination of melarsoprol in biological fluids by high-performance liquid chromatography and characterisation of two stereoisomers by nuclear magnetic resonance spectroscopy

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Abstract

The analysis of melarsoprol in whole blood, plasma, urine and cerebrospinal fluid is described. Extraction was made with a mixture of chloroform and acetonitrile followed by back-extraction into phosphoric acid. A reversed-phase liquid chromatography system with ultraviolet detection was used. The relative standard deviation was 1% at concentrations around 10 μmol/l and 3–6% at the lower limit of determination (9 nmol/l in plasma, 93 nmol/l in whole blood, 45 nmol/l in urine and 10 nmol/l in cerebrospinal fluid). Melarsoprol is not a stable compound and samples to be stored for longer periods of time should be kept at −70°C. Plasma samples can be stored at −20°C for up to 2 months. Chromatography showed that melarsoprol contains two components. Using nuclear magnetic resonance spectroscopy the two components were shown to be diastereomers which slowly equilibrate by inversion of the configuration at the As atom.

Keywords: Nuclear magnetic resonance spectroscopy; Melarsoprol

1. Introduction

Melarsoprol is the most frequently used drug for treatment of late stage *Trypanosoma gambiense* and *T. rhodesiense* sleeping sickness with involvement of the central nervous system. The drug is administered according to complicated dosage schedules during a total of three to four weeks, and the dosage recommendations vary. Side-effects are common, the most serious being encephalopathy, which occurs in 2 to 5% of the patients [1,2]. Other frequent side-effects are albuminuria and abdominal colic pain. Also nausea, vomiting, diarrhoea, dermatitis and arthralgia may occur.

The complicated dosage schedules are based on clinical experience only as the pharmacokinetics and metabolism of melarsoprol are unknown. There is no knowledge of relationships between drug concentrations and side effects, and nothing is known about the minimal parasiticidal concentrations in vivo.

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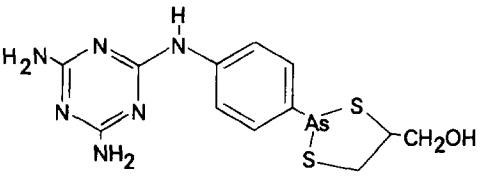
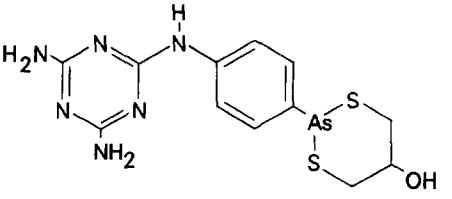
The safety of treatment with melarsoprol may be improved by optimising the drug regimen. Knowledge of the pharmacokinetics and relationship between drug concentrations and adverse effects is therefore essential. This requires determination of melarsoprol concentrations in biological fluids. An enzyme-linked immunosorbent assay (ELISA) has been published [3], but methods based on antibody reactions are not suitable for pharmacokinetic studies until their specificity has been clearly shown. A bioassay has also been developed [4], but this method measures trypanocidal activity and co-determines active metabolites. The high-performance liquid chromatography (HPLC) method described by Berger and Fairlamb [5] uses a gradient system and is obviously not intended for analysis of a series of samples in clinical studies. We therefore decided to develop an assay based on reversed-phase HPLC for determination of melarsoprol in plasma, whole blood, urine and cerebrospinal fluid.



Melarsoprol



Internal standard

Fig. 1. Structures of melarsoprol and the internal standard 2-[4-(4,6-diamino-s-triazin-2-yl-amino)phenyl]-5-hydroxy-1,3,2-dithiarsenane.

2. Experimental

2.1. Chemicals

Melarsoprol and the internal standard 2-[4-(4,6-diamino-s-triazin-2-yl-amino)phenyl]-5-hydroxy-1,3,2-dithiarsenane (Fig. 1) were obtained from Rhône-Poulenc Rorer (Vitry sur Seine, France). Solvents and chemicals (HPLC or analytical grade, as required) were purchased from Merck (Darmstadt, Germany).

Stock solutions of melarsoprol and the internal standard, usually 3 μ*M*, were prepared in dimethylformamide and diluted with phosphate buffer 0.1 *M*, pH 7, before use.

2.2. NMR spectroscopy[1]

NMR spectra of solutions in dimethylsulfoxide-d_6 were recorded at 80°C. Chemical shifts are reported in parts/million (ppm), using dimethylsulfoxide-d_6 (δ 2.62, ^{1}H; δ 39.6, ^{13}C) as reference. The two-dimensional DQF-COSY, two-dimensional TOCSY (both run in the phase sensitive mode), HMQC and DEPT experiments were performed on a JEOL EX-270 spectrometer (JEOL, Tokyo, Japan) with standard JEOL pulse sequences. The TOCSY experiment was performed with a mixing time of 120 ms.

2.3. HPLC–MS

HPLC–MS was run using a Finnigan TSQ 700 (Finnigan, San Jose, CA, USA) mass spectrometer. The liquid chromatograph was a Waters MS-silk system (Waters Associates, Milford, MA, USA) connected to the mass spectrometer by a Finnigan TSPII (thermospray) interface. The column and eluent were the same as described below in Section 2.4 except that ammonium acetate was used in the buffer.

2.4. Chromatographic system

The chromatograph consisted of a ConstaMetric 3500 pump (LDC, Riviera Beach, FL, USA), a

[1] The following abbreviations are used. DQF-COSY: double quantum filtered correlation spectroscopy; TOCSY: total coherence spectroscopy; DEPT: distortionless enhanced polarisation transfer; HMQC: heteronuclear multiple quantum coherence.

Rheodyne 7125 injector (Rheodyne, Berkeley, CA, USA), a Shimadzu SPD-6A UV detector (Shimadzu, Kyoto, Japan) and an LKB 2221 integrator (LKB, Bromma, Sweden). The detection wavelength was 283 nm and peak areas were measured. The column was an Ultrasphere ODS, 3 μm, 75×4.6 mm I.D. (Beckman, Berkeley, CA, USA). The eluent was prepared by mixing 320 ml 0.1 *M* sodium phosphate buffer pH 2 with 95 ml acetonitrile and adjusting the pH to 3 with 5 *M* NaOH. The flow-rate was 1 ml/min.

2.5. Samples

Samples for the development and evaluation of the method were prepared by adding known amounts of melarsoprol to blank whole blood, plasma, urine and cerebrospinal fluid. Samples were also obtained from patients during treatment for *T. gambiense* sleeping sickness in Daloa, Côte d'Ivoire. The blood samples were immediately centrifuged and all samples were stored at −70°C.

2.6. Analytical procedure

2.6.1. Plasma

Plasma samples, 1.0 ml, were pipetted into 5-ml polypropylene tubes. A 100-μl volume of 1 *M* phosphate buffer, pH 7, 50–100 μl of a 1.2, 30 or 300 μ*M* solution of the internal standard (the amount of internal standard was chosen to fit the concentration range of the standard curve) and 2 ml of chloroform–acetonitrile (60:40, v/v) were added. After mixing for 20 min using a rotating mixer, the tubes were centrifuged for 10 min at 2500 *g*. The organic (lower) phase was transferred to new tubes containing 300 μl of 0.1 *M* phosphoric acid. The tubes were shaken for 10 min and centrifuged as above and the organic phase was discarded. Then 50–150 μl of the aqueous phase was injected into the chromatographic system.

2.6.2. Whole blood

Whole blood samples, 200 μl, were pipetted into 5-ml polypropylene tubes. A 1-ml volume of 0.1 *M* phosphate buffer, pH 7, 50–100 μl of a 1.2, 30 or 300 μ*M* solution of the internal standard and 2 ml of chloroform–acetonitrile (60:40, v/v) were added. After mixing and centrifuging as above, the organic phase was transferred to new tubes and then treated as described for plasma samples.

2.6.3. Urine

Urine samples, 200 μl, were pipetted into 5-ml polypropylene tubes. A 1-ml volume of 0.1 *M* phosphate buffer, pH 7, 50–100 μl of a 1.2, 30 or 300 μ*M* solution of the internal standard and 2 ml of the chloroform–acetonitrile mixture were added. The samples were then treated as described for plasma.

2.6.4. Cerebrospinal fluid

Cerebrospinal fluid samples, 1 ml, 100 μl of 1 *M* phosphate buffer, pH 7, 50 μl of a 1.2 or 30 μ*M* solution of the internal standard and 2 ml of the of chloroform–acetonitrile mixture were added. The samples were then treated as described for plasma.

2.7. Standard curves

Standard curves were prepared by adding known amounts of melarsoprol to drug-free plasma, whole blood, urine and cerebrospinal fluid. The standard samples were treated as described above. The amount of the internal standard was 0.1 to 30 nmol, depending on the concentration range of the standard curve. The resulting peak-height ratios were plotted vs. the concentrations.

2.8. Extraction recovery

Melarsoprol was added to plasma, whole blood, urine and cerebrospinal fluid ($n=4$, Table 1). Extraction was done as described above, and peak heights were compared with those of directly injected standards.

2.9. Precision, accuracy and limit of determination

Known amounts of melarsoprol were added to plasma, whole blood, urine and cerebrospinal fluid. The concentrations are given in Table 1. The samples were analysed as described above. The within-assay R.S.D. was determined using ten replicates.

Table 1
Within-assay reproducibility, accuracy and extraction recovery ($n=10$)

Sample (sample volume)	Added concentration	Mean concentration found	R.S.D. (%)	Extraction recovery (mean±S.D., $n=4$) (%)
Plasma (1 ml)	11.3 μmol/l	11.9 μmol/l	1	
	635 nmol/l	668 nmol/l	2	86±3
	9.1 nmol/l	9.1 nmol/l	6	
Whole blood (200 μl)	11.3 μmol/l	12.4 μmol/l	1	
	635 nmol/l	627 nmol/l	3	34±1
	93.2 nmol/l	117 nmol/l	2	
Urine (200 μl)	11.3 μmol/l	12.0 μmol/l	1	
	635 nmol/l	645 nmol/l	2	75±2
	45.4 nmol/l	44.6 nmol/l	3	
Cerebrospinal fluid (1 ml)	101 nmol/l	91.4 nmol/l	2	82±4
	10.1 nmol/l	10.9 nmol/l	6	

The inter-assay R.S.D. was determined using one single sample in each run.

2.10. Stability

Melarsoprol was added to drug-free plasma, whole blood and urine to obtain concentrations of 600–850 nmol/l. The samples were stored at room temperature, 4, −20 and −70°C. The concentration was determined at intervals (Table 2) during 2 weeks for the samples stored at room temperature, 1 month for the samples at 4°C and 6 months for the samples at −20 and −70°C.

2.11. Interferences

Samples from patients treated with chloroquine, pentamidine, phenobarbital, carbamazepine and phenytoin, as well as samples spiked with quinine, sulphadoxine, pyrimethamine and paracetamol, were analysed as described above.

3. Results

3.1. Chromatograms

Chromatograms of extracts of samples containing melarsoprol are shown in Fig. 2. Melarsoprol gives rise to two peaks with a peak-area ratio of 3:1. LC–MS showed that the two components had identical mass spectra with a molecular ion corresponding to the molecular mass of melarsoprol plus one hydrogen. Also, the internal standard gives rise to two peaks and contains two isomeric components. The broad peak eluting about 13 min after injection is due to an unidentified endogenous compound.

3.2. NMR assignments

^{1}H and ^{13}C NMR chemical shifts of melarsoprol are given in Table 3. The ^{1}H and ^{13}C NMR spectra reflected the fact that melarsoprol existed in two forms since major and minor signals in the ratio 3:1 were observed. The ^{13}C NMR spectrum of melarsoprol showed inter alia signals at δ 42.06, 42.78, 58.70, 62.08, 62.96 and 63.21. A 90° DEPT experiment clearly identified the signals at δ 58.70 (major) and 62.02 (minor) to correspond to methine groups. The remaining signals at δ 42.06, 42.78 and 62.96, 63.21 thus correspond to C-3 and C-1, respectively (Fig. 3). Signals for aromatic carbons are observed at δ 119.17, 130.54, 134.01, 141.61, 164.68 and 167.09. The ^{1}H NMR spectrum showed in the downfield region, signals for aromatic protons at δ 7.38 [0.3 H, doublet (d), $J_{H,H}$ 8.6 Hz], 7.35 (1H, d, $J_{H,H}$ 8.6 Hz), 7.05 (0.3 H, d, $J_{H,H}$ 8.6 Hz,) and 7.00 (1H, d, $J_{H,H}$ 8.6 Hz,). The DQF-COSY spectrum confirmed connectivities of signals at δ 7.38 to 7.05 and 7.35 to 7.00, respectively. The high field region of the ^{1}H NMR spectrum showed signals at δ 4.40 (1 H, broad, J not resolved), 3.48 [1H, multiplet (m)],

Table 2
Stability of melarsoprol in biological samples during storage

Days	Room temperature	4°C	−20°C	−70°C
Plasma				
0	636	636	636	636
1	524	667	712	690
2	391	667	703	706
3	296	703	718	714
7	55	609	678	675
14		525	663	683
30		382	738	766
60			651	829
90			459	816
120			432	807
180			228	728
345			87	840
Whole blood				
0	854	854	854	854
1	696	888	869	871
2	567	811	864	996
4	384	635	664	773
7	295	577	536	785
16	132	247	377	726
30		166	181	790
60			100	829
120			99	729
180			129	760
330			78	637
Urine				
0	606	606	606	606
2	112	819	884	833
3	69	551	661	652
7		492	707	690
14		431	624	600
21		313	635	657
30			755	789
60			389	433
90			481	537
180			710	762
350			489	623

2.85 (2H, m), 3.26 [1H, double doublet (dd), $J_{H,H}$ 12.5 and 4 Hz] and 2.46 (1H, dd, $J_{H,H}$ 12.5 and 4.3 Hz). Signals for the minor component are observed at δ 4.45 (0.3H, broad), 3.12 (0.3H, dd, $J_{H,H}$ 12.8 and 4.3 Hz), 2.4 (0.3H, dd, $J_{H,H}$ 12.5 and 8.9 Hz) as well as in the region from δ 3.20–2.84 (H, J not resolved). Connectivities of all protons could be obtained in 2D DQF-COSY and TOCSY spectra. Correlation of the protons with their corresponding carbons was obtained in coupled HMQC spectra. Since no C–H correlation from the signals at δ 4.40 and 4.45 was observed, these signals are assigned to correspond to the hydroxyl group.

3.3. Reproducibility, standard curves, stability and interferences

Table 1 shows the within-assay reproducibility, accuracy and extraction recovery for spiked samples. The inter-assay R.S.D. for 1-ml plasma samples containing 638 nmol/l was 6% ($n=11$).

The standard curves were linear within the concentration ranges that were tested, i.e. 10 nmol/l to 30 μmol/l, and the correlation coefficients have been 0.991 or better. The inter-assay R.S.D. for the slope of the standard curve for 1-ml plasma samples was found to be 8% ($n=11$).

The stability of melarsoprol in biological samples during storage is shown in Table 2. At room temperature, a decrease in concentration was noticed already after 5 h. Plasma samples are stable for 2 months and urine samples for at least 1 month at −20°C. At −70°C the melarsoprol concentration remained unchanged during one year.

When samples from patients treated with sulphadoxine and pyrimethamine (Fansidar) were analysed, two peaks were found to interfere with the peaks M1 and IS1. No other interferences were observed by the drugs that were tested.

4. Discussion

4.1. NMR investigation of isomeric forms of melarsoprol

In solution, two isomeric forms of melarsoprol equilibrate to the ratio 3:1. The most likely explanation for this is the pyramidal structure of trivalent arsenic, which slowly inverts to give the two diastereomers A and B (Fig. 3).

The ^{13}C NMR signal for C-2 of the major and the minor compound resonate at δ 58.70 and 62.08, respectively. This chemical shift difference of 3.38 ppm is significant, whereas the signals for C-1 and C-3 only show minor differences and the aromatic carbons have identical chemical shifts for the major and the minor compound. A chemical shift difference of the magnitude observed for C-2 is, in

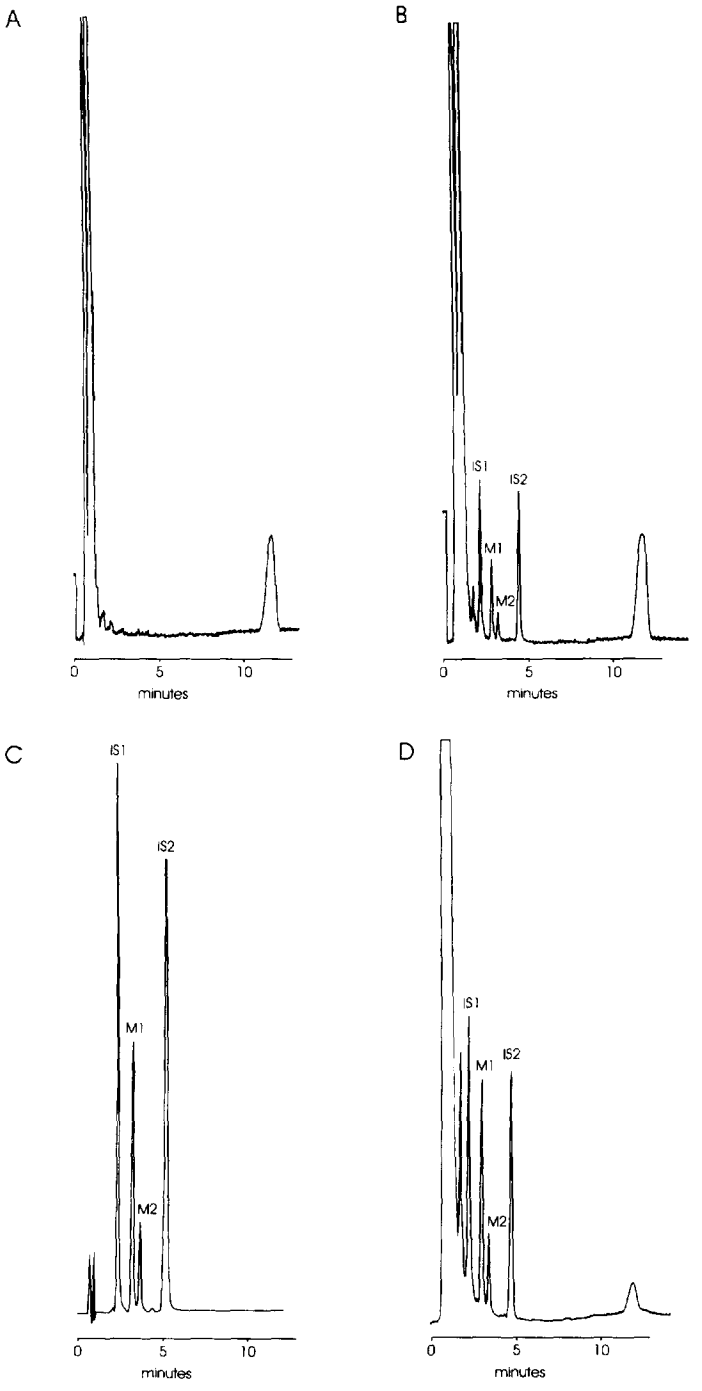


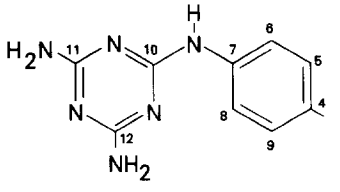
Fig. 2. Chromatograms of melarsoprol samples. (A) Blank plasma. (B) Plasma containing internal standard (IS1 and IS2) and 9.1 nmol/l melarsoprol (M1 and M2). (C) Plasma sample from a patient. The melarsoprol concentration is 101 nmol/l. (D) Urine containing internal standard and 635 nmol/l melarsoprol.

Table 3
The ^{13}C and ^{1}H NMR chemical shifts[a] of melarsoprol isomers A and B, obtained at 80°C

A, major component					B, minor component				
Position	Chemical shift (ppm)	Position	Chemical shift (ppm)	Coupling constant (Hz)	Position	Chemical shift (ppm)	Position	Chemical shift (ppm)	Coupling constant (Hz)
C-1	62.96	H-1	2.85	N.r.[b]	C-1	63.21	H-1	~2.84–3.28	N.r.
		H-1′	2.85	N.r.			H-1′	~2.84–3.28	N.r.
C-2	58.70	H-2	3.48	N.r.	C-2	62.08	H-2	~2.84–3.28	N.r.
C-3	42.06	$H\text{-}3_a$	2.46	4.0, 12.5	C-3	42.78	$H\text{-}3_a$	2.40	8.9, 12.5
		$H\text{-}3_e$	3.26	4.3, 12.5			$H\text{-}3_e$	3.12	4.3, 12.5
C-6,8	119.17	H-4	7.35	8.6	C-6,8	119.17	H-4	7.38	8.6
C-5,9	130.54	H-5	7.00		C-5,9	130.54	H-5	7.05	
C-4	134.01	H-6			C-4	134.01	H-6		
C-7	141.61	H-7			C-7	141.61	H-7		
C-10	164.68	H-8			C-10	164.68	H-8		
C-11,12	167.09	H-9			C-11,12	167.09	H-9		

[a] Chemical shifts are given in ppm relative to d_6-DMSO, 39.6 ppm (^{13}C) and 2.60 ppm (^{1}H) for solutions in d_6-DMSO.
[b] N.r.: not resolved.
Protons of NH and OH for A are identified at δ 5.57 and 4.40, respectively.
Protons of NH and OH for B are identified at δ 8.22 and 4.45, respectively.

general, seen for a substituted carbon in a six-membered ring when the substituent changes from an axial to an equatorial position. This should also be true for five-membered rings. The lower chemical shift value of C-2 of the major compound thus implies a conformation of the five-membered ring where the CH_2OH group adopts a pseudoaxial position, whereas in the minor component the CH_2OH group is in a pseudoequatorial position. It is thus concluded that inversion at arsenic even alters the conformation of the five-membered ring.



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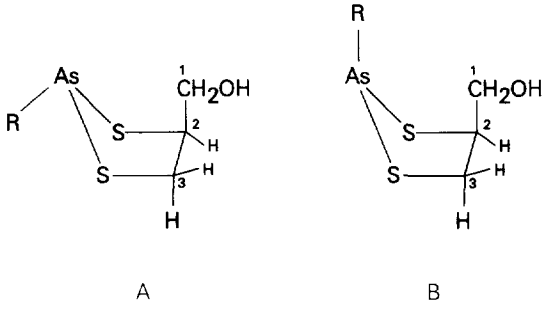
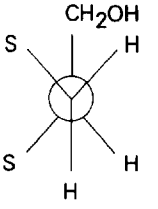


A B

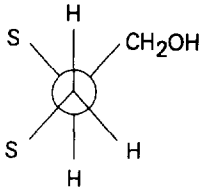
Fig. 3. Conformation of the melarsoprol isomers.

$H\text{-}3_{axial}$ and $H\text{-}3_{equatorial}$ of the major compound show a geminal coupling of 12.5 Hz and vicinal couplings of 4.0 and 4.3 Hz. According to Karplus this indicates a constellation of $H\text{-}3_{axial,equatorial}$ and H-2 as shown in 1:



1

$H\text{-}3_{axial}$ and $H\text{-}3_{equatorial}$ of the minor compound show a geminal coupling of 12.5 Hz and vicinal couplings of 8.9 and 4.3 Hz. According to Karplus this indicates a constellation of $H\text{-}3_{axial,equatorial}$ and H-2 as shown in 2:



2

This additionally confirmed that the major compound has a predominant ring conformation where the CH_2OH group is axially oriented.

It is reasonable to assume that a pseudoaxial orientation of the CH_2OH group is favoured if R is *trans* to the CH_2OH group, as in Fig. 3A. In addition, an equatorial orientation should be favoured if R is *cis* to the CH_2OH group. We thus conclude that the major compound is A and the minor is B (Fig. 3B does not show the preferred conformation).

4.2. Chromatography

The sum of peaks M1 and M2 is used for quantitation, but it is also possible to use only M2 if necessary. This is the case when the patient has been treated with sulfadoxine and pyrimethamine (Fansidar), which gives rise to interferences with M1 and IS1. The internal standard peak IS2 is always used. Use of M2 for quantitation does not significantly affect the performance of the assay, except that the lower limit of determination will be marginally higher. None of the other drugs that were tested interfered with the determination. A broad peak, which is due to an unknown endogenous compound, appears 13 min after injection in chromatograms of extracts of plasma, whole blood and urine.

The nature of IS1 and IS2 has not been studied in as much detail as the melarsoprol peaks. However, IS1 and IS2 show identical mass spectra, and NMR spectra indicate that they are conformational isomers in analogy with melarsoprol.

4.3. Extraction recovery

The extraction of melarsoprol presents some problems since the drug is rather unstable in aqueous solution. Melarsoprol is a base, but an increase of the pH to 8 or more results in rapid hydrolysis. The procedure that we have chosen, extraction at pH 7 with a mixture of chloroform and acetonitrile, gives clean chromatograms and good extraction recoveries from all media, except for whole blood (Table 1). The low recovery from whole blood may be due to binding of the drug to cells. Haemolysis by dilution with water might have improved the recovery, but this was not tested since the reproducibility of the assay was excellent despite the low recovery.

4.4. Precision, accuracy and limit of determination

The precision and accuracy are excellent, despite the low extraction recovery and the instability of the drug. One reason for the good performance of the method is that the internal standard, an isomer of melarsoprol, is ideal. The lower limit of determination, defined as the lowest concentration that can be determined with an intra-day R.S.D. of less than 10% and acceptable accuracy, was 9 nmol/l for 1-ml plasma samples. The separation of the phases during extraction is difficult when 1-ml whole blood samples are extracted; therefore 200-μl samples were used. The lower limit of determination will therefore be higher than for plasma, 93 nmol/l. Urine gives rise to a broad front peak which interferes with the determination if 1-ml samples are used. Samples of 200 μl can be analysed without problems with a lower limit of determination of 45 nmol/l.

4.5. Stability

Melarsoprol is not a stable compound, and we recommend storage of samples at −70°C unless they are going to be analysed immediately (Table 2). If necessary, plasma samples can be kept at −20°C for up to two months, whereas the melarsoprol concentration in whole blood samples decreases already after 2 days at this temperature. Storage in a refrigerator is possible only during a working day. The ratio between the two diastereomers does not change during storage.

Solutions of melarsoprol and the internal standard in anhydrous solvents can be stored for several months in the refrigerator. Stock solutions were therefore prepared in dimethylformamide and diluted to the desired concentration immediately before use.

4.6. Patient samples

A small number of plasma, urine and cerebrospinal fluid samples from patients in Daloa, Côte d'Ivoire, have been analysed. Plasma concentrations of 2–15 μM were found immediately after the fourth dose (1.2, 2.4, 3.6, 3.6 mg/kg) of melarsoprol. No interfering peaks were observed in the chromatograms of the patient samples. The metabolic fate of melarsoprol is not known and no peaks that were suspected to be due to metabolites were found.

Acknowledgments

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