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Rapid, highly sensitive gradient narrow-bore high-performance liquid chromatographic determination of suramin and its analogues

Matthias Kassack, Peter Nickel*

University of Bonn, Pharmaceutical Institute, An der Immenburg 4, D-53121 Bonn, Germany

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Abstract

A high-performance liquid chromatography (HPLC) method for the determination of suramin, its precursors and analogues in aqueous solutions and in plasma samples with advantages compared to earlier methods is described. Due to the method's high sensitivity (detection limit of suramin in plasma samples: 7 ng/ml; in aqueous solutions: 5 ng/ml) and selectivity (suramin t_R: 7.05 min, precursor amine **2** t_R: 4.68 min), it is possible to analyze degradation products, impurities and possible metabolites of suramin besides suramin. Tetrabutylammonium hydrogensulfate (TBAHS) (5 m*M*) is used as ion-pairing reagent in a mixture of 36% methanol and 0.02 *M* phosphate buffer pH 6.5 is used as the mobile phase. After sample injection, a linear gradient from 36 to 62.9% methanol is run. A C_8 stationary phase (100×2.1 mm I.D.) is used and ultraviolet (UV) detection at 238 nm is applied. Plasma extraction is performed with tetrabutylammonium bromide (pH 8.0) and acetonitrile. This procedure allows the determination of suramin and its precursor amine **2** in the range of 0.05–400 μg/ml with high precision [relative standard deviation of peak areas at 0.05 μg/ml: 2.10% (n=5)] and nearly complete recovery (>96.5%). Because of the high flexibility of the chromatographic system and subsequently the universality of the method, the analysis of a broad range of suramin analogues is possible. The result of the purity check of two suramin analogues is given.

Keywords: Suramin

1. Introduction

In 1920 suramin (Germanin), a polysulfonated naphthylurea (Fig. 1) was introduced by Bayer in the therapy of African trypanosomiasis [1]. About 30 years later Van Hoof discovered the antifilarial activity of suramin [2]. In 1979 De Clercq reported suramin to be a potent inhibitor of the reverse transcriptase of retroviruses and to have an anti-retroviral activity in vitro [3]. Subsequent clinical trials to evaluate the efficacy of suramin in the treatment of acquired immune deficiency syndrome (AIDS) yielded disappointing results [4]. But these investigations indicated that suramin has an anti-tumour activity. Suramin inhibits the proliferation of several cancer cell lines at concentrations easily achievable in the plasma of patients. Furthermore, suramin can induce the differentiation of tumour cells [5,6].

Clinical trials with advanced prostate and adreno-cortical cancer patients showed promising results (tumour/metastasis regression), considering the severity of the illness and the lack of therapeutic

*Corresponding author.

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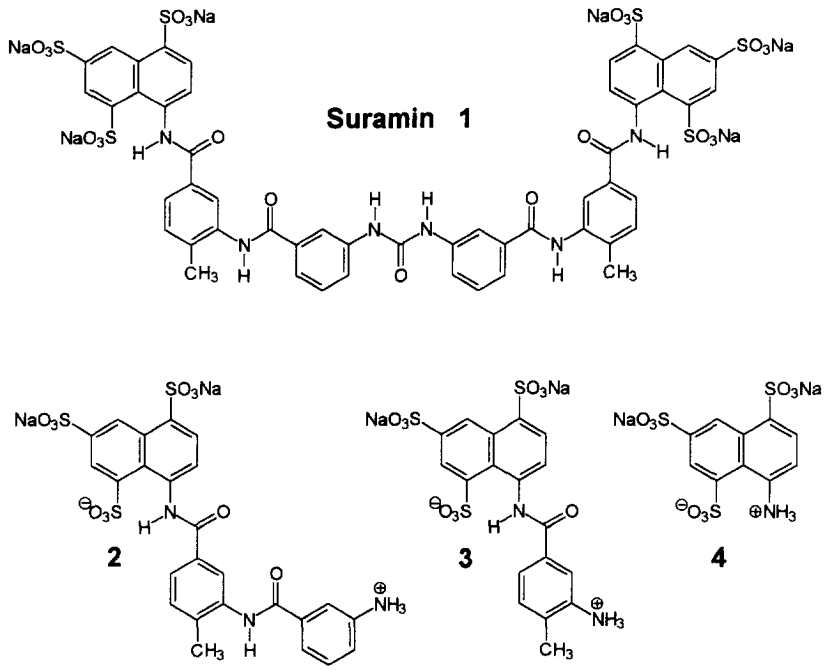

Fig. 1. Suramin and its synthetic precursors.

alternatives [7–9]. A thorough pharmacokinetic monitoring is necessary to secure therapeutic effective plasma levels and to minimize severe side effects. The plasma levels should be between 200 and 300 μg/ml [10,11].

A large series of suramin analogues was synthesized in our research group. Several analogues showed interesting biological activities: antifilarial activity [12,13], inhibition of the reverse transcriptase of HIV [14], antagonistic activities at P_2 purinoceptors [15–17], inhibition of ecto ATPase [18], induction of cell differentiation of human colon cancer cells [19,20].

Several HPLC methods for the determination of suramin in plasma have been described [6]. However, no method is available for the identification and determination of potential suramin metabolites, precursors or degradation products besides suramin. Furthermore, the purity of our suramin analogues which are biologically tested has to be proven. Therefore, we developed this sensitive, selective and flexible HPLC method.

2. Experimental

2.1. Chemicals

Suramin (**1**, Germanin) and 1-aminonaphthalene-4,6,8-trisulfonic acid (**4**) were supplied by Bayer (Leverkusen, Germany). The compounds **2**, **3**, **5** and **6** were synthesized by Nickel et al. [12,31] (see Fig. 1 for structures of the compounds). Methanol was of gradient grade (LiChrosolv Merck, Darmstadt, Germany). The ion-pair reagent tetrabutylammonium hydrogensulfate (TBAHS) was of higher purity than the corresponding bromide (TBAB) and purchased at Serva (Heidelberg, Germany). The phosphate buffer reagents were of analytical grade (Fluka, Buchs, Switzerland). Freshly distilled water was of sufficient purity and used as such. TBAB analytical grade (Fluka), used for the plasma extraction procedure was purified by pumping a solution through a C_{18} reversed-phase column. Acetonitrile for protein denaturation was of HPLC grade.

2.2. Apparatus

HPLC was performed on a Hewlett-Packard (HP) chromatography system (HP, Waldbronn, Germany): HP 1050 quaternary pump equipped with a manual 20-μl loop injector Rheodyne 7125 (Rheodyne, Cotati, CA, USA), diode array detector HP 1040 A. Recording and processing of detector signals were performed on a HP computer 9000 Series 300 Model 310 with the HP 79994 A HPLC ChemStation.

2.3. Chromatography

Analytical column: HP 100×2.1 mm I.D., guard column: HP 20×2.1 mm I.D., both filled with MOS Hypersil 5 μm (RP-8). The column temperature was 40°C. The capillaries were of 0.12 mm I.D. The eluent was a mixture of 80% A and 20% B. A was a mixture of 100.0 ml of methanol and of 400.0 ml of 0.02 *M* phosphate buffer (pH 6.5) containing 6.25 mmol/l of TBAHS. B was 100% methanol. Linear gradient conditions were as follows: 0 min: A=80%, B=20.0% (equivalent to 36.0% methanol); 0–8 min: A=46.4%, B=53.6% (equivalent to 62.9% methanol); 8–9 min: A=80%, B=20.0% (equivalent to 36.0% methanol).

A flow-rate of 0.6 ml/min was maintained. UV detection at 220 and 238 nm was applied. The reference wavelength was 550 nm. UV spectra were stored peak controlled.

2.4. Purity check of suramin analogues

Aqueous solutions with a concentration of about 100 μg/ml were prepared. Aliquots of these solutions (10 μl) were injected into the liquid chromatography (LC) system. A peak purity check was performed by overlaying UV spectra from the upslope, the apex and the downslope of a peak. The conformity of the spectra was stated by the match factor which corresponds to the least square fit coefficient. A match factor of 0 indicates no match and one of 1000 indicates identical spectra [32,33]. The purity of a suramin analogue is given in area percent after integration of all peaks of a chromatogram. For the main peak and the detected impurities identical absorption coefficients were assumed.

2.5. Plasma extraction procedure

A 250-μl volume of a plasma sample was mixed with 250 μl of 1 *M* tetrabutylammonium bromide (TBAB) solution (pH 8.0) and shaken for 30 s. After addition of 500 μl of acetonitrile and shaking for 30 s, the samples were stored at 4°C for at least 2 h. Then, the samples were centrifuged at 3000 *g* for 10 min. The supernatant solutions were filtered and 20 μl of the filtrate were injected into the LC system.

3. Results

3.1. Chromatography

A rapid, sensitive, selective and flexible HPLC method for the determination of suramin as well as its precursors, degradation products and analogues has been developed and validated. Fig. 2 shows the chromatogram of an aqueous sample containing suramin and its amine precursors (cf. Fig. 1). The four compounds are well separated and elute with rising molecular mass (t_R: 7.04, 4.68, 4.14, 1.53 min for suramin, **2**, **3**, **4**, respectively).

The sensitivity of the method has been increased by using a gradient and a narrow-bore column. Fig. 3 shows three chromatograms using the same sample and injection volume, respectively. The chromatograms differ in using an isocratic or a gradient method (chromatograms a and b) and in using a column with 4 or 2.1 mm I.D. (chromatograms b and c). As Fig. 3 demonstrates, it is evident that the combination of the gradient and the narrow-bore method is much more sensitive than the isocratic method and about three times more sensitive than the gradient method.

The precision of the HPLC method appears to be good, as stated by five-fold analysis of samples containing suramin at various concentrations (Table 1).

The concentration of the organic modifier methanol in the eluent strongly influences the retention time of suramin. Therefore, great care should be taken in preparing eluent A. Observed variations of the retention times (cf. Table 1 and Table 2) are

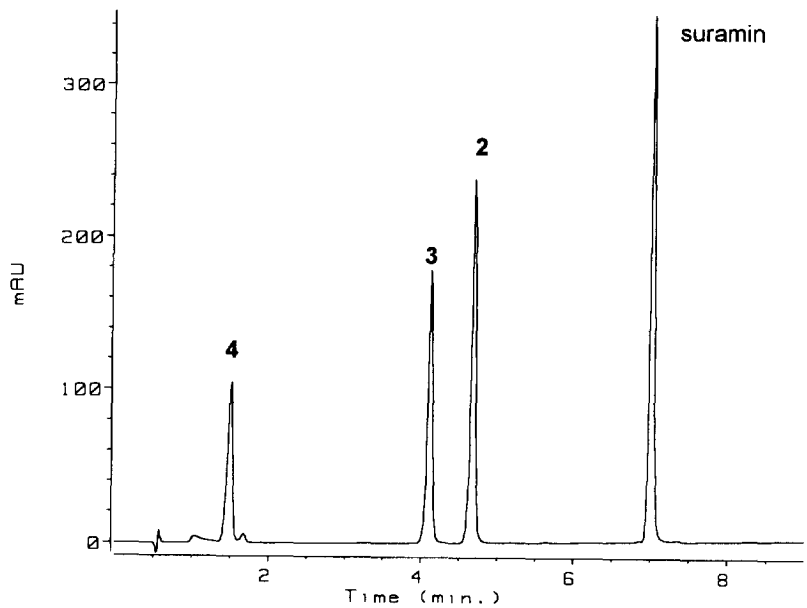

Fig. 2. Chromatogram of an aqueous solution of suramin and its three amine-precursors, 20 μg/ml, respectively.

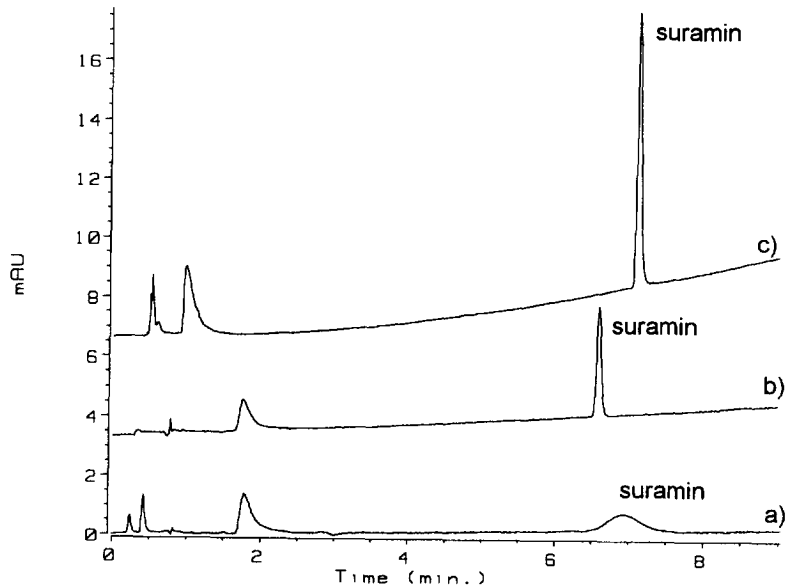

Fig. 3. Chromatograms of a solution of suramin (1.0 μg/ml); injection volume, 10 μl. (a) Isocratic method: column, 125×4 mm I.D. (RP C 8-silica); eluent, 2.5 mmol TBAB dissolved in a mixture of 480 ml methanol and 560 ml 0.02 *M* phosphate buffer pH 7.0; flow-rate, 1.5 ml/min. (b) Gradient method: column and flow-rate, as for (a); eluent and gradient conditions, as described in Section 2. (c) Gradient narrow-bore method: described in Section 2.

Table 1
Precision and accuracy of the suramin assay, mean values of five intra-day assays with the same charge of eluent A

Theoretical concentation of suramin (μg/ml)	t_R		Peak	
	Mean (min)	R.S.D. (%)[a]	Mean area	R.S.D. (%)
0.05	7.113	0.13	1.24	2.10
0.10	7.107	0.09	3.30	2.37
0.50	7.094	0.13	16.74	1.20
1.00	7.096	0.11	36.42	0.42
10.00	7.096	0.08	410.85	0.28
50.00	7.082	0.10	2034.60	0.73
100.00	7.092	0.10	4199.00	0.41

[a] R.S.D.(%): relative standard deviation in %.

assumed to be partially due to small variations of the methanol content of eluent A.

Due to fluctuating laboratory temperature, especially in summer, and subsequently fluctuating retention times, the column has to be thermostated. A temperature of 40°C was chosen which was above the highest room temperature observed in the laboratory, which is not air-conditioned. Furthermore, working at a column temperature of 40°C had the advantage that the same retention times (compared to a column temperature of 20°C) could be achieved with a lower methanol content in the eluent at the start of the gradient. Under the chosen chromatographic conditions, the retention times are reliable parameters of identification, as shown for suramin in Table 2. Furthermore, the identification power can be increased by comparing the UV spectrum of a compound with spectra of reference compounds.

TBAHS was used instead of TBAB because of its higher purity. The TBAHS concentration strongly influenced the retention time/capacity factor: a logarithmic correlation between capacity factor and the TBAHS concentration was observed. Chromatograms were stored and evaluated at 238 nm, the absorption maximum of suramin. No interference with plasma peaks (cf. Fig. 4) or other peaks (injection event, solvent impurities, cf. Fig. 3) was observed. The second universal detection wavelength, 220 nm, was used to detect impurities and degradation products in different samples.

Suramin and its precursors **2**, **3** and **4** are stable in aqueous solution at room temperature at least for 40 days when stored protected from light [34].

Table 2
Retention times of suramin, intra-day and inter-day precision mean values of intra-day ($n=4$) and inter-day ($n=5$) assays with the same charge of eluent A

Day	Mean t_R ($n=4$)	R.S.D. (%)
Intra-day		
1	6.695	0.24
2	6.686	0.21
3	6.664	0.17
6	6.727	0.05
8	6.696	0.01
Inter-day ($n=5$)	6.694	0.30

3.2. Plasma extraction

The analysis of a spiked plasma sample of suramin and its amine precursor **2** is depicted in Fig. 4b. There is no interference between compound peaks and plasma components (cf. Fig. 4a). Plasma components elute between 9 and 13 min while purging with methanol. Purging with methanol (conditions cf. Fig. 4) is not absolutely necessary. However, it increases the lifetime of the column to more than 150 injections. Without purging, about 150 injections can be run with the above stated precision. One run takes 9 min (conditions described in Section 2).

TBAB (1 *M*, pH 8.0) and acetonitrile were used for plasma extraction. TBAB had to be purified. TBAHS could not be used because of the desired pH. A pH>7 was chosen because of the pH dependence of the suramin binding to human albumin: the higher the pH the lower the binding [28]. Albumin was shown to have three binding sites for suramin at pH 6.0, two with lower affinity at pH 7.4 and only one at pH 9.2. Using TBAB solutions with a pH>8 was avoided because at a higher pH the silica-based Hypersil stationary phase could be damaged. As shown in Fig. 5, the recovery of suramin and its amine precursor **2** depends on the concentration of TBAB. The amine precursor **2** can be extracted much more easily from plasma than suramin. This difference between the two compounds can be easily explained. The plasma protein binding mainly depends on the number of sulfonic acid groups just like the persistence of suramin and analogues in the blood [29,30]. When performing the plasma extrac-

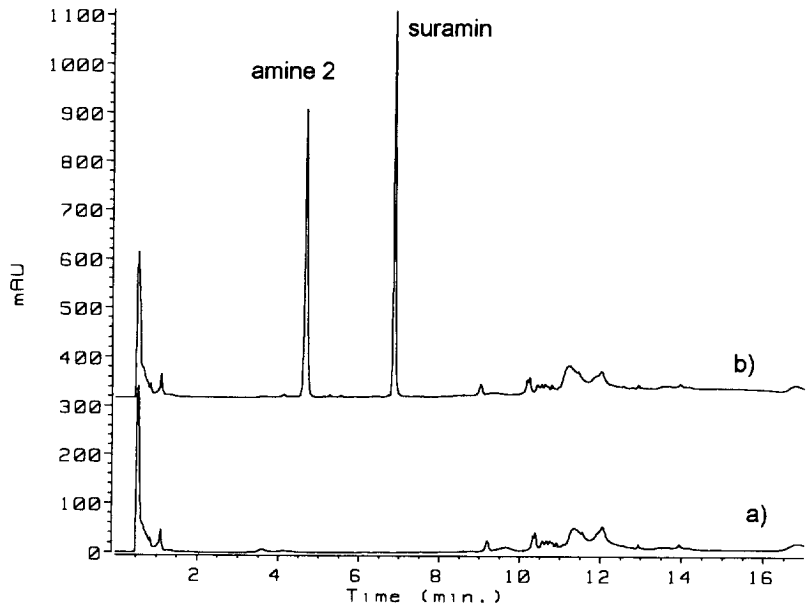

Fig. 4. Chromatogram of (a) plasma blank and (b) plasma sample containing suramin and its amine-precursor **2**, 100 μg/ml, respectively. Purge conditions (8–17 min): 8–12 min, gradient to 100% methanol; 12–14 min, 100% methanol; 14–17 min, gradient to initial conditions (A=80%, B=20%).

tion without TBAB the recovery of suramin is about 65% but the recovery of amine **2** is still 92.3% (cf. Fig. 5). Using a 1 *M* TBAB solution ensures nearly complete recovery. The rate of recovery also depends on the plasma and on the concentration of the compounds in the plasma, as Table 3 shows. The extraction of plasma of four different donators spiked with 100 μg/ml suramin and amine **2**, respectively, yielded recoveries between 95.9 and 98.4% for suramin and between 94.8 and 97.9% for amine **2** (cf. Table 3). In the concentration range of 0.05–400 μg/ml, a recovery of >96.5% for both compounds could be achieved with a high precision, apart from the lowest concentration of 0.05 μg/ml for suramin (cf. Table 4). A more complete protein denaturation was achieved by using acetonitrile instead of methanol and by storage of the protein acetonitrile mixture at 4°C for at least 2 h.

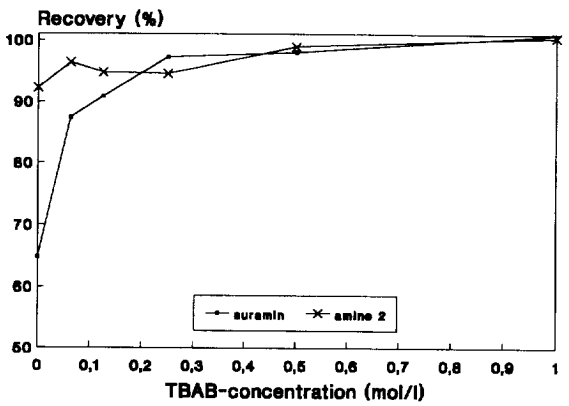

Fig. 5. Influence of the TBAB concentration in the solution used for the plasma extraction on the recovery of suramin and amine **2**.

Table 3
Recovery of suramin and amine **2** from plasma or blood. Dependency on the type of plasma or blood

Matrix	Suramin		Amine **2**	
	Mean recovery (%)	R.S.D. (%)	Mean recovery (%)	R.S.D. (%)
Plasma 1	97.1	1.30	97.1	1.30
Plasma 2	95.9	1.87	94.8	1.94
Plasma 3	97.1	1.65	96.5	1.66
Plasma 4	98.4	1.01	97.9	1.07
Blood 1	97.5	1.25	91.9	1.25
Blood 2	96.7	1.53	91.7	1.55

Theoretical concentration 100 μg/ml (suramin or amine **2**, respectively). Values represent % mean recovery (n=7).

Table 4
Dependency of the rate of recovery of suramin and amine **2**, respectively, on the concentration of the compounds in a pooled plasma

Theoretical concentration (μg/ml)	Suramin		Amine **2**	
	Mean recovery (%)	R.S.D. (%)	Mean recovery (%)	R.S.D. (%)
0.05	94.8	2.45	100.3	6.97
0.10	102.1	0.97	98.4	5.63
0.25	98.7	2.03	99.1	0.84
0.50	100.9	1.20	101.0	0.05
1.00	99.6	0.42	100.0	0.79
2.50	99.4	0.27	98.2	0.47
5.00	99.3	1.14	98.2	0.57
10.00	99.9	0.21	98.4	0.47
25.00	101.4	0.52	100.5	0.28
50.00	99.3	0.20	98.6	0.24
100.00	100.6	1.32	99.5	0.81
200.00	98.3	0.55	98.5	0.70
300.00	101.0	2.60	99.4	1.94
400.00	101.9	1.93	99.8	1.11

Values represent % mean recovery ($n=3$).

3.3. Quantification of suramin and amine 2

The detection limits of suramin/amine **2** in aqueous samples are 5/7 ng/ml, in plasma samples 7/10 ng/ml, respectively (signal-to-noise ratio=2.5:1). The determination of suramin and amine **2** in plasma is possible in the concentration range of 0.05 to 400 μg/ml. To achieve reliable results when determining suramin/amine **2** samples in this large concentration range, two calibration curves had to be constructed: one with the peak areas of the low concentration range (0.05–1 μg/ml), another one with the peak areas of the whole concentration range (0.05–400 μg/ml). The resulting low concentration calibration curve was used between 0.05 μg/ml and the point of intersection of the two calibration curves (here 7.5 μg/ml). The high concentration calibration curve was used from the point of intersection (7.5 μg/ml) to 400 μg/ml. The calibration curves of suramin could be characterized as follows (mean of 3 estimations): low concentration range (0.05–7.5 μg/ml), peak area=29.473 [±0.155]×concentration (μg/ml) −0.413 [±0.033], $r=1.0000$; high concentration range (7.5–400 μg/ml), peak area=30.420 [±0.103]×concentration (μg/ml) − 6.969 [±0.314], $r=0.9998$.

As shown by the correlation coefficient r, the linearity of the results was good.

3.4. Purity check of suramin analogues

The high flexibility of the developed HPLC method allows the direct analyses of many suramin analogues, as shown for the analysis of compound **5** (Fig. 6 and Fig. 7) [12]. Besides the main peak (**5**, $t_R=5.9$ min), the zoom shows two small peaks at $t_R=1.4$ min and 3.8 min. Using reference compounds, by comparison of the retention times and UV spectra, the two small peaks could be shown to be the amine precursors **4** and **3** of **5**. To verify there was no impurity peak hidden under the main peak, the purity of the main peak (compound **5**) was confirmed by overlaying the peak spectra of the upslope, the apex and the downslope, and subsequent yield of a match factor of 1000. Considering the impurity peaks at $t_R=1.4$ min and 3.8 min, the purity of compound **5** is about 98.5%.

The HPLC method also allows a fast modification of the concentration of the organic modifier methanol in the eluent. Therefore, when analysing suramin analogues which are not well separated from their impurities under the chromatographic conditions described in Section 2, the selectivity can be quickly improved. An example shows the analysis of compound **6** (the purification of **6** caused some difficulties [31]), (cf. Fig. 6 and Fig. 8). The methanol concentration and gradient conditions were quickly

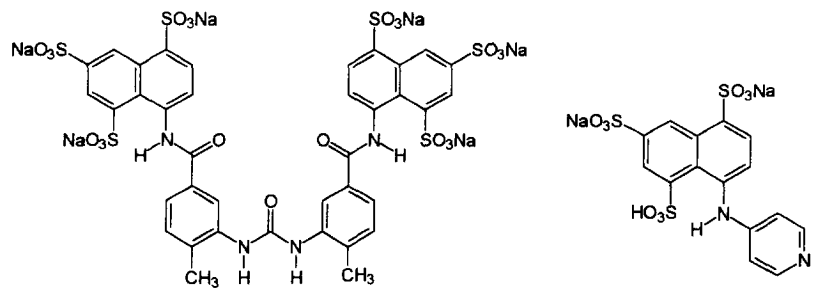

Fig. 6. Structures of compounds **5** and **6**.

varied as follows: 0 min, A=90%, B=10% (equivalent to 28% methanol); 0–8 min, A=53%, B=47% (equivalent to 57.6% methanol); 8–9 min, A=90%, B=10% (equivalent to 28% methanol).

The result is a chromatogram that shows a main peak with t_R=1.98 min (compound **6**) well separated from its impurity peaks (t_R: 4.05 and 4.74 min, respectively). Under standard chromatographic conditions (cf. Section 2 the main peak showed a shoulder and was insufficiently separated from its impurities.

4. Discussion

A HPLC method for the determination of suramin, its precursors, degradation products and analogues in aqueous solution and in plasma samples is described. So far there was no method available for the parallel determination and identification of potential suramin metabolites, precursors or degradation products besides suramin, or for the determination of suramin analogues. As Fig. 2 shows, the described HPLC method has a high selectivity for the parallel de-

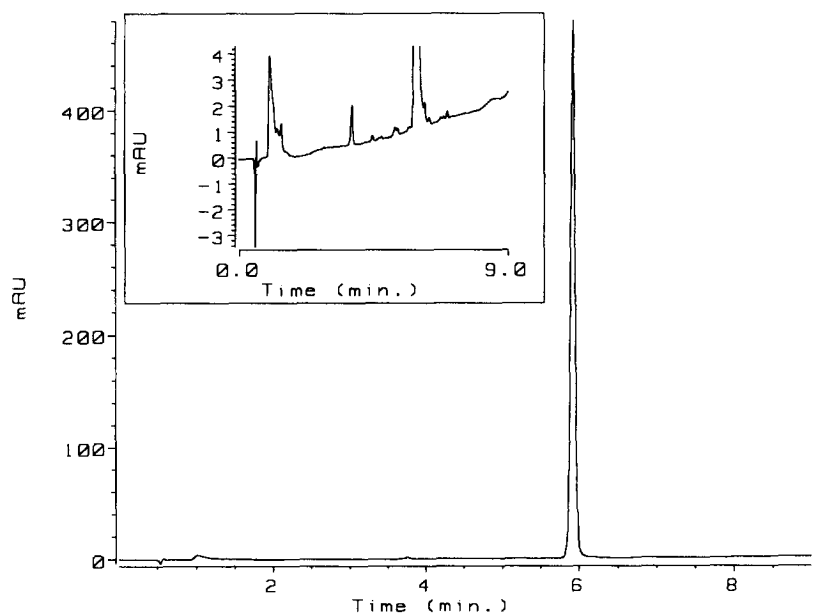

Fig. 7. Chromatogram of a sample of compound **5** (100 μg/ml). The insert shows a zoom of the same chromatogram to detect small peaks apart from the main peak.

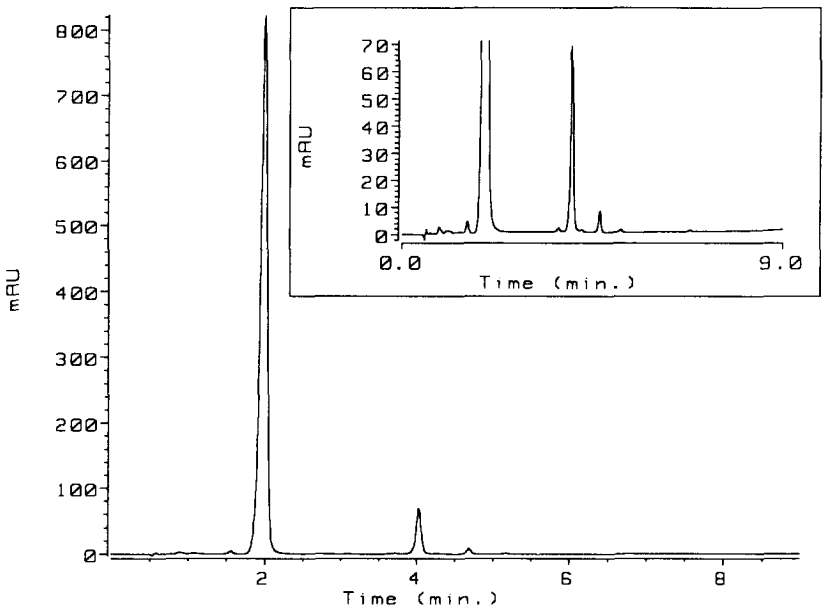

Fig. 8. Chromatogram of an aqueous solution of compound **6** (100 μg/ml). Gradient conditions: see text. The insert shows a zoom of the same chromatogram to detect small peaks apart from the main peak.

termination of precursors besides suramin. Some of the previously published HPLC methods have a too low selectivity for the separation of suramin and its precursors because of disturbing solvent-, plasma-, or internal standard-peaks [11,22–27]. The combination of a gradient elution and a narrow-bore column (2.1 mm I.D.) increased the sensitivity several-fold compared to the use of an isocratic elution [22,27] or standard chromatographic columns with 4 mm I.D. (Fig. 3). Thus, the described HPLC method is highly sensitive, as shown by the detection limits of suramin and its precursor amine **2** in plasma (7 and 10 ng/ml, respectively). Considering the therapeutic drug levels of suramin (200–300 μg/ml) [10,11] the sensitivity is sufficient for a pharmacokinetic monitoring and a search for potential metabolites of suramin. Coleman has demonstrated the existence of biliary metabolites in an experiment with ^{14}C labelled suramin in the isolated perfused rat liver [21]. But he did not identify the chemical structure of the metabolites. Now, due to the availability of reference compounds [14] and a determination method for these precursors and potential metabolites of suramin, an identification of the previously found metabolites in the rat liver seems to be possible.

As shown by the high precision of the method and the high recovery rates of suramin and amine **2** from plasma and blood (cf. Table 3 and Table 4), a quantification is possible without using an internal standard, an advantage compared to several previous methods. This results in less work and produces a chromatogram without a disturbing internal standard peak, which could hide an impurity or metabolite of suramin or its analogues.

The use of a narrow-bore column as well as the thermostating of the column at 40°C resulted in a decreased use of methanol. However, performing the chromatography at 20°C is possible. Then the methanol concentration has to be increased, to achieve the same retention times for suramin or its analogues obtained when working at 40°C.

The universality and flexibility of the HPLC method is based on the possibility of a very quick variation of the content of the organic modifier methanol in the eluent and shown by the analysis of two suramin analogues (Fig. 7 and Fig. 8).

Compounds, which are evaluated for their biological activity, have to be of high chemical purity. The HPLC purity check of our suramin analogues includes two steps: detecting impurity peaks besides

the main compound peak and secondly, checking the purity of the main peak. To minimize the chance of not detecting an impurity, which elutes at a similar retention time as the main peak, the peak spectra of upslope, apex and downslope of the main peak are measured and compared. A peak yielding a match factor of >995 (identical spectra) is considered as pure. Now, with this method the purity of our biologically tested compounds can be guaranteed.

The accuracy of the identification of a peak can also be dramatically increased by the combination of comparing retention times and UV spectra. Even between compounds with very similar spectra a discrimination is possible, e.g. suramin and its precursor amine **2**, not regarding their different retention times (data not shown). This high identification power will be helpful in identifying potential suramin metabolites.

The described HPLC method has advantages compared to previously published methods, especially in terms of a parallel analysis of suramin and its precursors and degradation products. The flexibility of the method allows the purity check of suramin analogues. Presently undiscovered metabolites could be detected in biological fluids of patients treated with suramin by using this sensitive and selective method. Pharmacokinetic monitoring of suramin or of its analogues is possible.

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