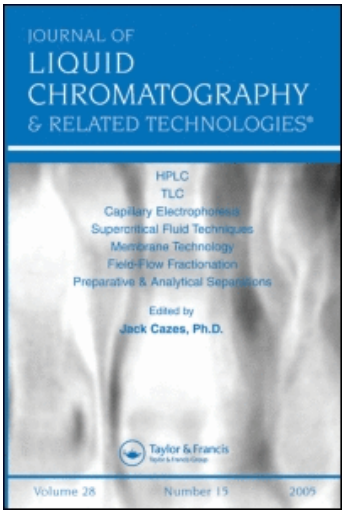

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QUANTIFICATION OF SURAMIN BY REVERSE-PHASE ION-PAIRING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A specific and sensitive method has been developed for the separation and quantification of suramin and trypan blue (internal standard) in human plasma. Plasma samples were extracted by centrifugation after the addition of ion-pairing reagent (tetrabutylammonium phosphate, TBAP) and methanol. Extracts were injected directly onto a reverse-phase ion-pairing HPLC system with 5 mM TBAP in the mobile phase. There was nearly 100% extraction efficiency after 3 cumulative extracts of each sample. The limit of quantitation was 0.5 μg/ml at a detection wavelength of 313 nm. Analysis of 3 post-therapy samples from a patient with AIDS was used to determine a plasma half-life for suramin of at least 3 weeks.

INTRODUCTION

Suramin (hexasodium salt of 8,8'-(carbonylbis(imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene)carbonylimino)) bis-1,3,5-napthalenetrisulfonic acid, Fig. 1a) was introduced in

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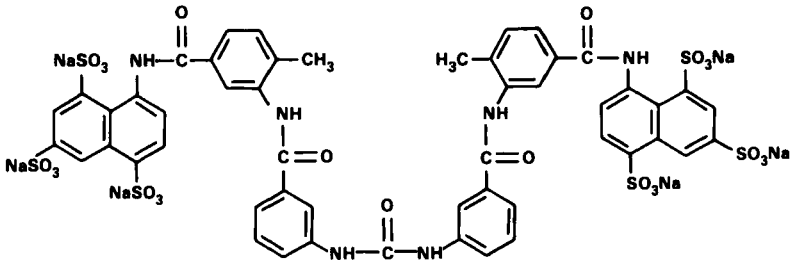
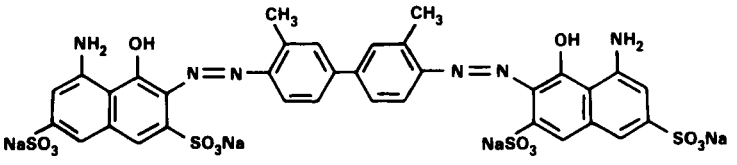
a



SURAMIN

b



TRYPAN BLUE

Figure 1. Structures of (a) suramin and (b) trypan blue.

1920 and has been used in the therapy of human trypanosomiasis and onchocerciasis (1). More recently, it has started trials in humans for the treatment of Acquired Immunodeficiency Syndrome (AIDS). Broder and Gallo have reviewed the relationship between AIDS and human T-cell leukemia virus (HTLV) (2). The pathogenesis of AIDS is thought to be linked to the retrovirus referred to as HTLV-III. Retroviruses are RNA viruses that require an enzyme known as reverse transcriptase for their propagation (3,4). Suramin inhibits the action of reverse transcriptase and protects T-cells against infectivity and the cytopathic effect of HTLV-III (5,6).

Early pharmacology studies of suramin were performed using non-specific analytical techniques (7,8). These methods required lengthy hydrolysis and other reaction steps to obtain a derivatized product suitable for colorimetric determination of suramin. This paper presents a new analytical method for the measurement of suramin in plasma by reverse-phase ion-paired HPLC.

MATERIALS AND METHODS

Reagents

Suramin was obtained from the Developemental Therapeutics Program of the National Cancer Institute (Bethesda, MD). Trypan blue was from Grand Island Biological Co. (Grand Island, NY). Tetrabutylammonium phosphate (TBAP) was obtained from Eastman Kodak Co. (Rochester, NY). The ammonium acetate and glacial acetic acid were ACS grade from Fisher (Fairlawn, NJ). The methanol was HPLC grade and was also from Fisher. Double-distilled water was used for all solution preparations.

High-Performance Liquid Chromatography

Chromatographic separation was acheived on a radial-pak, C_{18}, 10 μm column in a radial compression system (Z-module, Waters Associates, Milford, MA). Peak detection was accomplished on a Waters model 440 fixed wavelength detector at 313 nm. A 1 M ammonium acetate solution was adjusted to pH 6.55 with glacial acetic acid and was used in the preparation of the solvents for liquid chromatography. TBAP was added with ammonium acetate to water and passed through a 0.45 μm filter before the addition of methanol. Solvent "A" contained 5 mM TBAP, 10 mM ammonium acetate, and 30% methanol (v/v). Solvent "B" contained 5 mM TBAP, 10 mM ammonium acetate, and 90% methanol (v/v). Initial conditions were 40% pump "B" at a flow of 2 ml/min. Each analytical run included a 10-min linear gradient from 40% pump "B" to 70% pump "B". An 8-min equilibration delay was allowed before the next sample injection.

Extraction Procedure

Each 0.5 ml plasma sample received 50 µl of trypan blue (200 µg/ml) as internal standard (Fig. 1b). Then, 100 µl of a 0.5 M TBAP solution was added followed by 1 ml of methanol. The samples were mixed vigorously (Vortex-Genie, Scientific Industries, Bohemia, NY) and centrifuged at 2000 rpm for 5 min. The supernatant was decanted into another tube. Second aliquots of 100 µl 0.5 M TBAP and 1 ml methanol were added to the pellet, mixed vigorously, and centrifuged. The supernatant was decanted into the same tube as the first extract. A third cycle of TBAP plus methanol was repeated for each sample. The extract pool was mixed and centrifuged at 2000 rpm for 5 min. 50 µl of the supernatant was injected onto the HPLC.

Standard Curves and Extraction Recovery

Standard curves in plasma were prepared by adding aliquots of stock aqueous suramin solutions to human plasma obtained from a normal volunteer. Standards were prepared in triplicate for concentrations from 0.5 µg/ml to 300 µg/ml. Each triplicate 0.5 ml standard was taken from a common spiked plasma solution. Single standards of 50, 100, 200, 400, 800 and 1200 µg/ml were also extracted.

The recovery of suramin (50 µg/ml) and trypan blue (20 µg/ml) for each cumulative extraction was determined from triplicate 0.5 ml plasma samples.

Patient Samples

Suramin (6.2 g) was administered in divided doses over a 5-week period. Blood samples were collected in heparinized tubes and obtained on days 1, 7, and 28 after the last suramin dose. Plasma was separated by centrifugation and stored frozen until analysis.

Since the biological samples were obtained from patients with AIDS, additional care was taken in handling the blood and plasma.

All patient sample manipulations were confined to a class II biological safety hood (9). Samples were enclosed in protected centrifuge chambers during centrifugation outside of the safety hood.

Calculations

The standard curve data were fit to the straight-line equation, $y = mx + b$, where m was the slope of the line and b was the y-intercept. The fit was performed with MLAB, a curve-fitting program (10). The y-value was the peak height ratio (PHR) of suramin/trypan blue. The x-value was the known concentration of suramin. The weighting factor was $1/(PHR)^2$.

RESULTS

Plasma Extracts

Figure 2 shows the chromatograms of extracts of normal human plasma. The chromatogram was void of peaks at retention times greater than 2 min for extracts of normal human plasma. Trypan blue eluted first at 7 min followed by suramin 1.5 min later (Fig. 2b). The methanol gradient was required to give a single sharp peak for suramin. The amount of methanol in the initial conditions determined the resolution of suramin. An increase in the initial methanol resulted in the elution of two peaks at different retention times for suramin.

The purity of trypan blue should be determined before its use as an internal standard. Trypan blue has been used primarily as a biological stain, for which an analytical grade is not required for most investigators. Some sources of trypan blue included a contaminant which gave a peak with the identical retention time as suramin (data not shown).

Standard Curve

The standard curve was linear from 0.5 μg/ml to 100 μg/ml (Fig. 3). Peak height ratios for concentrations between 100

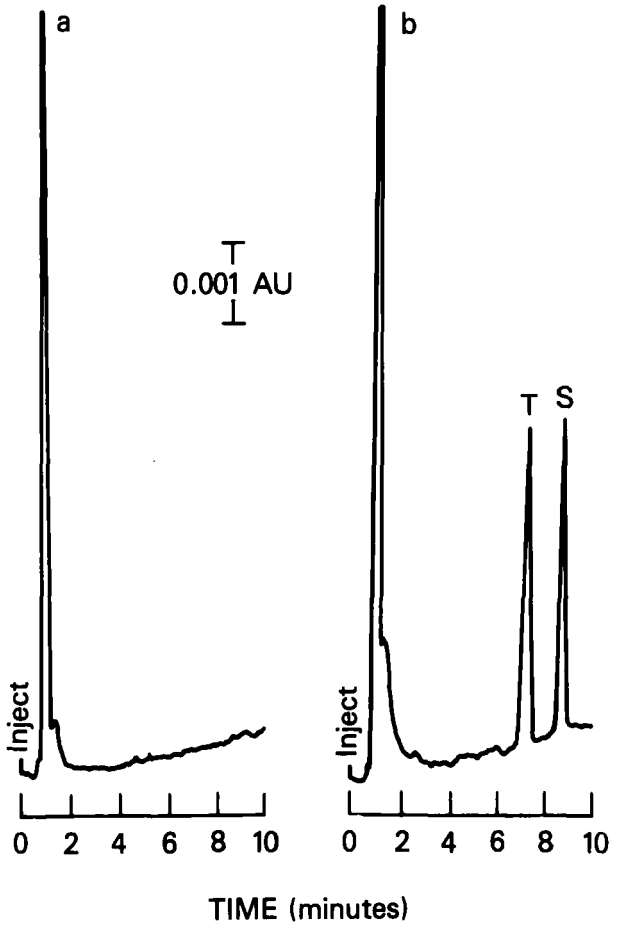


Figure 2. Chromatograms for 0.5 ml samples of (a) normal human plasma and (b) normal human plasma with 20 µg/ml trypan blue (T) and 10 µg/ml suramin (S). 50 µl of each extract were injected onto the HPLC with detection at 313 nm and attenuation of 0.01 absorbance units (AU) full-scale.

TABLE 1

Accuracy and Precision of Suramin Standard Curve

Suramin added (μg/ml)	Suramin recovered (μg/ml)	Suramin recovered (% added)
0.5	0.534 ± 0.03*	107 ± 6*
1.0	0.919 ± 0.05	92 ± 5
2.5	2.51 ± 0.1	100 ± 5
5.0	4.97 ± 0.2	100 ± 3
10	10.3 ± 0.3	103 ± 3
25	25.4 ± 1	102 ± 4
50	51.2 ± 2	102 ± 4
100	98.3 ± 0.8	98 ± 1
200	190 ± 3	95 ± 1
300	267 ± 9	89 ± 3

* Mean and standard deviation

μg/ml and 1200 μg/ml began to deviate from a straight line by curving downward. The curvature was observed regardless of whether peak height or peak area was used to determine the ratio of suramin to trypan blue. Dose solutions of equivalent suramin concentrations displayed a curvature similar to that of the extracted plasma standards.

The lower limit of quantitation for the analysis was approximately 0.5 μg/ml. The peak height of the 0.5 μg/ml standard was only 3% of the full-scale absorbance of 0.01 absorbance units. However, this peak height was still more than 5 times the baseline noise.

Only the standards with concentrations of 0.5 μg/ml to 100 μg/ml were used to generate the line of best fit for the data. Table 1 includes the data generated from analysis of the standard curve. It shows the concentration of suramin calculated from the PHR of each standard including the 200 μg/ml and 300 μg/ml standards. Deviation from the calculated standards was greatest at the extremes of the standard curve. The downward curvature

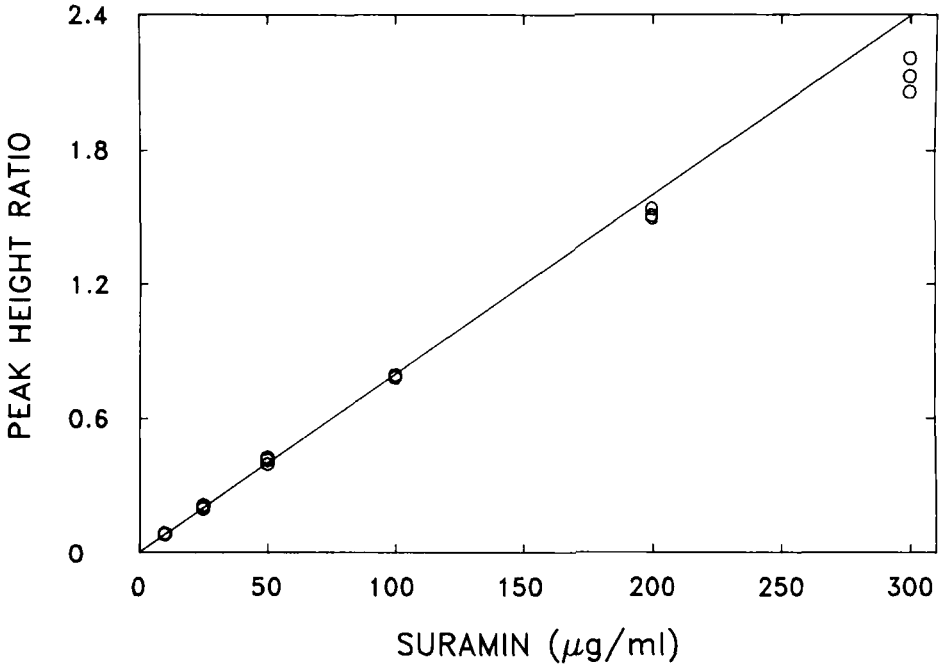


Figure 3. Standard curve of suramin (triplicates) in normal human plasma. The circles are the individual PHR measurements at 313 nm at attenuations of 0.1 for suramin and 0.01 for trypan blue. The solid line represents the weighted-fit of the standards from 0.5 μg/ml to 100 μg/ml.

at the higher concentrations has already been described (Fig. 3). Precision in measurement for triplicate samples gave a maximum variation of 6% in the 0.5 μg/ml standard (limit of quantitation). The variation in the remaining samples was less than 6% (Table 1).

Recovery

Three cumulative extractions of plasma samples spiked with 20 μg/ml trypan blue and 50 μg/ml suramin were sufficient to acheive greater than 95% recovery for each compound (Table 2). Two cumulative extractions gave a recovery of 90% and 84% for trypan blue and suramin, respectively.

TABLE 2

Extraction Recovery for Suramin and Trypan Blue

Number of cumulative extracts	Trypan Blue (%Recovery)	Suramin (%Recovery)
1	66 ± 3*	54 ± 1*
2	90 ± 1	84 ± 3
3	95 ± 2	97 ± 1

* Mean and standard deviation

Hydrolysis of suramin was performed at 95°C for 1 hour in either 0.1 M HCl or 0.1 M NaOH (data not shown). The combined results of the hydrolyses gave at least 6 other products as determined by the presence of 6 additional peaks in the HPLC chromatogram at 254 and 313 nm. One of the 6 peaks co-eluted with the internal standard trypan blue. Recovery of the hydrolytic products from extracted human plasma was similar to the extraction recovery of suramin in plasma.

Patient Samples

Following the last dose, the disappearance of suramin from plasma of a single patient is shown in Figure 4. The 3 samples indicate that the half-life of suramin in plasma is greater than 21 days.

DISCUSSION

The analysis procedure for suramin is simple and sensitive. Although it requires a triple extraction of each sample to insure nearly 100% recovery, the method is much less laborious than previously-reported methods. Because suramin is highly protein bound (1), it was felt that there would be more confidence in the patient data when the triple extraction scheme was used. The limit of quantitation of the current method is one-tenth that of

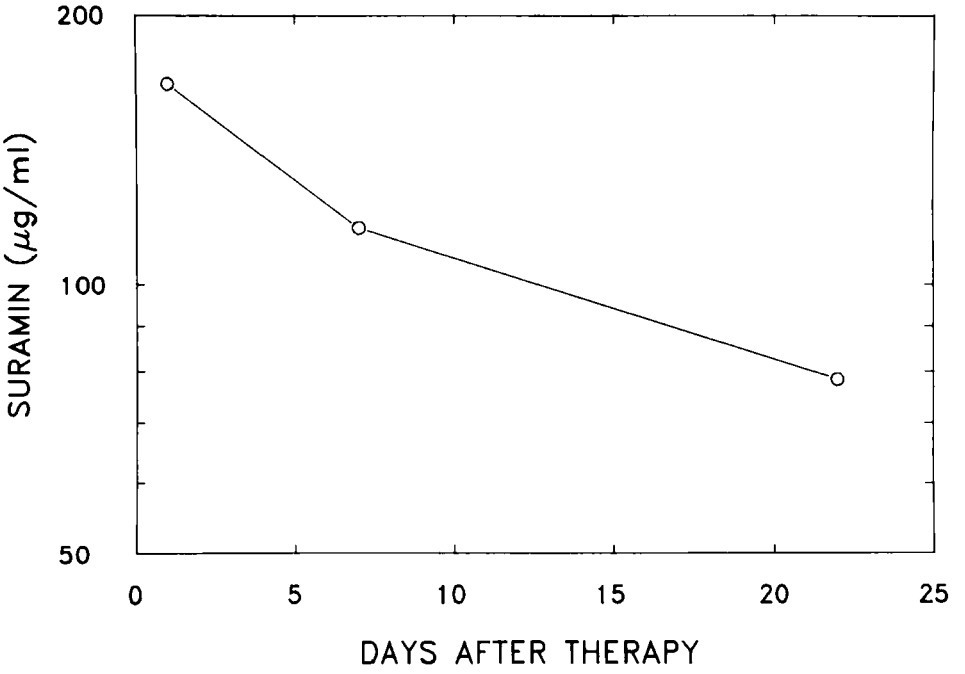


Figure 4. Disappearance of suramin from plasma of a single patient following the last dose.

the background noise of other methods(7,8). Further improvements in assay sensitivity by concentration of the extractant has not been explored. In addition to sensitivity, this method provides specificity. Other methods required hydrolysis of suramin prior to derivitization for colorimetric determination. At least six potential metabolites obtained from mild hydrolysis of suramin can be separated from suramin in this HPLC method. Possible metabolic products of suramin would be included in the suramin measurement of the previous methods.

There are three areas of caution to be noted in the use of this analysis procedure. First, additional care in sample handling is required due to the nature of the patients' disease, AIDS, in the present study. Second, careful attention should be

paid to the source and purity of the internal standard, trypan blue. Different sources and manufactured batches from the same source have varied in their purity. Finally, if concentrations above 100 µg/ml to 200 µg/ml suramin are expected in patient samples then the standard curve should be adequately represented in that concentration range. Alternatively, the more concentrated samples could be diluted into the linear region of the standard curve.

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