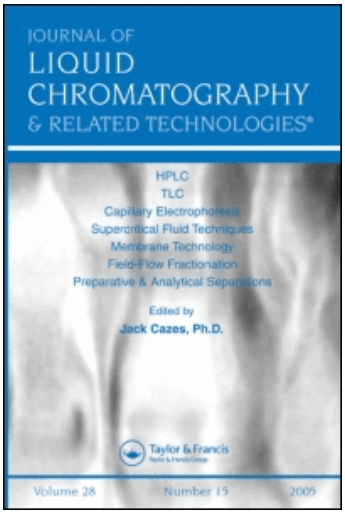

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Determination of Suramin in Plasma and Urine by Ion-Paired Reverse-Phase High-Performance Liquid Chromatography

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DETERMINATION OF SURAMIN IN PLASMA AND URINE BY ION-PAIRED REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-pressure liquid chromatographic (HPLC) assay has been developed for the quantification of the anti-viral drug suramin (SUR) in human plasma and urine. This assay is simple and accurate, using an isocratic mobile phase in a reverse-phase ion-pairing mode.

This assay was developed for the determination of high levels of suramin in the urine and plasma of patients being treated for the acquired immunodefficiency Syndrome (AIDS). The limit of detection of this assay was 0.25 µg/mL. Congo red (CR) was used as the internal standard with a retention time of 5.9 min, while suramin had a retention time of 13 min.

INTRODUCTION

Before the onset of the acquired immunodeficiency syndrome (AIDS), suramin was used solely for the treatment of parasitic diseases such as trypanosomiasis

and onchocerciasis. Recently, however, suramin has been found to inhibit in vitro the cytopathic effects of the human immunodeficiency virus (HIV), a virus believed to be etiologically associated with AIDS (1). This finding has led to investigations of the safety and efficacy of this agent for the treatment of patients with this disorder. Recent studies have indicated that suramin may be of limited use in this role as single agent therapy (2).

Unfortunately, little is known regarding the pharmacokinetic disposition of suramin. Hawking (3), has previously attempted to study the distribution and elimination characteristics of this agent, but these results have been limited by the use of colorimetric assay methods that are less specific than more conventional assays using high-pressure liquid chromatography (HPLC). The HPLC assay for suramin developed by Klecker and associates(4)has problems with reproducability and internal standard, and we found this method to be limited. Recently, Ruprecht et.al. (5) published an assay using an isocratic mobile phase, but the internal standard requires a triple recrystalization process before its use. Furthermore, this assay measures drug concentrations in plasma samples only.

We have developed a simple HPLC assay for determining suramin levels in both plasma and urine.

EXPERIMENTAL

Apparatus

Two separate systems were used demonstrating the adaptability of the assay to varying systems and components.

System I consisted of a Beckman 112 pump (Beckman Instruments Inc., Berkeley, CA), a Waters WISP 710B automatic injector (Waters Associates, Milford, MA) and a Waters model 441 UV detector. The detector wavelength was set at 254 nm, and the signal evaluated at an absorbance of 0.1 AUFS. Peak integration was recorded with a Hewlett-Packard 3392A integrator (Hewlett-Packard Co., Avondale, PA), and the column used was a Waters Nova-Pak C_{18}, 4μ(3.9 mm x 15 cm).

System II utilized a Waters M-45 pump, U6K manual injector(a Waters WISP 710B was used when multiple samples were run) , and a model 481 UV detector, set at

a wavelength of 254 nm, 0.1 AUFS (all Waters) . The column was a Waters Nova-Pak C_{18}, 4μ(3.9mm x 15cm), and detected peaks recorded with a Hewlett-Packard 3392A integrator.

Reagents

All water used in the assay was de-ionized through a "nanopure" water filtration system (Millipore, Milford, MA).

The reagents used were : Acetonitrile (CH_3CN), HPLC grade, obtained from Fischer Scientific (Pittsburg, PA) ; sodium suramin stock from Mobay Chemical Corp., made by FBA Pharmaceuticals (Fig.1a; lot HU971K, New York, NY); congo red, Fischer Scientific, Chemical Manufacturing Division (Fig.1b; lot 745785, Fairlawn, NJ) ; ammonium acetate (NH_4OAc ,lot 02607 HM) , and tetrabutylammonium chloride (TBACl, lot 4904TL) both from Aldrich Chemical Co. (Milwaukee, WI) ; and glacial acetic acid (HOAc), Mallinckrodt Inc. (lot 7739 KAGY, Paris, KY). All reagents were used as recieved.

Stock Solutions

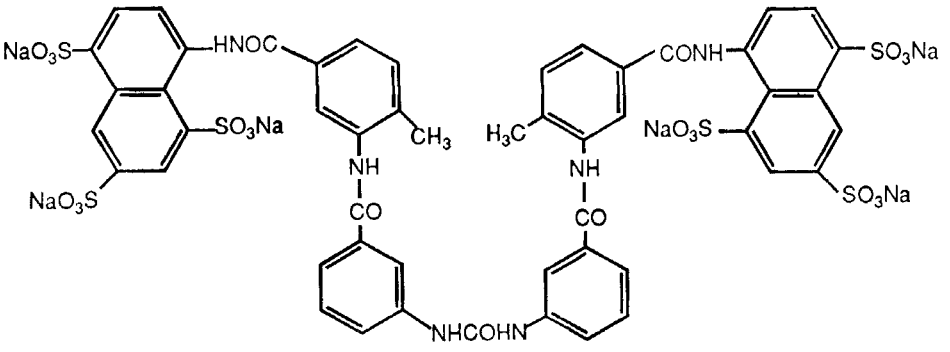
The stock solutions used were stable until time of use and made as follows:

SUR stock- 10mg suramin powder/ 5mL "nanopure" H_2O
CR stock- 8mg CR/ 5mL "nanopure" H_2O to dissolve, brought up to 50mL with CH_3CN.
TBACl (0.5 M) stock- 0.5 M TBACl in "nanopure" H_2O.
NH_4OAc/TBACl stock (50 mM NH_4OAc, 26 mM TBACl)- 50 mM NH_4OAc, 26 mM TBACl in "nanopure" H_2O (usually made in 2L quantities).

Mobile Phase

The mobile phase consisted of 42% CH_3CN, 10% NH_4OAc/TBACl stock, final pH at 6.8 and was prepared in two liter volumes using the following procedure: Eight hundred milliliters of CH_3CN was added to a 2L graduated cylinder, followed by enough "nanopure" water to bring up the volume to 1700mL. Next, 200mL of NH_4OAc/TBACl stock was added, and the final volume adjusted to 2L with "nanopure" water. This was then filtered under vacuum with a 0.45μm membrane filter, and the final pH adjusted to 6.8 with glacial acetic acid/ "nanopure" H_2O (1:3). The mobile phase was then pumped through the

a



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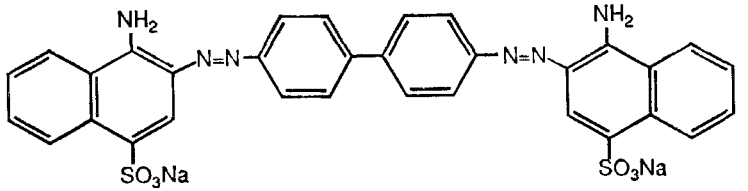

Figure 1: The chemical structures of a) Sodium Suramin, molecular weight 1429.21, and b) Congo Red, molecular weight 696.67.

column at a flow rate of 1.0mL/min., at a pressure of 1500 psi. The column equilibration time was about 30 min. before analysis could be initiated.

Sample Preparation and Analysis

Sample preparation required using a triple extraction method to assure complete separation of the drug from plasma proteins. In a test tube, 0.5mL of human plasma was added followed by the drug stock spike (for standard curve preparation). Next, 50µL of CR stock, the

internal standard, was added, followed by 100µL of TBACl (0.5M) stock and then vortexed for 15 sec. One milliliter of CH_3CN was added and vortexed to precipitate plasma proteins, and centrifuged at 3000 x g for 10 min. The resulting supernatant was then decanted and saved.

For the second extraction, 100µL of TBACl (0.5M) stock and 1mL of CH_3CN were added to the plasma pellet, the pellet was resuspended with a metal spatula, and vortexed. This was then centrifuged as before, and the supernatant was pooled with that from the first extraction. The third extraction was identical to the second, and the pooled supernatant was evaporated under a stream of N_2 to a volume of 1mL. Twelve microliters of the sample were then injected into the HPLC system.

The preparation of urine standard curves and clinical samples required a single extraction only. To 0.5mL of human urine, the SUR stock was spiked (for standard curve preparation), 50µL of CR stock was added followed by 1mL of CH_3CN and then vortexed. The samples were then ready for analysis.

RESULTS

Separation

Figures 2a-c show typical chromatograms for spiked human plasma samples representing concentrations of 0, 2, and 150µg/mL SUR. Figure 2d shows the chromatogram of a typical sample from an AIDS patient . A plasma level of 153.1µg/mL was measured following several months of therapy with 1 gram of suramin administered weekly. The internal standard's (CR) retention time was 5.9 min., while the retention time of SUR was 13 min. A large peak appearing at approximately 10 min. was an endogenous plasma peak present in both healthy volunteers' blank plasma used in standard curve preparation, and in the plasma of patients participating in the clinical study. Although always present, it did not interfere with either CR or SUR.

Linearity

Table 1 and figure 3 show the linear relationship between the concentration of SUR in plasma and the peak height ratio of SUR/CR . Using an unweighted method of least squares with a floating intercept and the origin as a data point, a coefficient of determination (r^2)

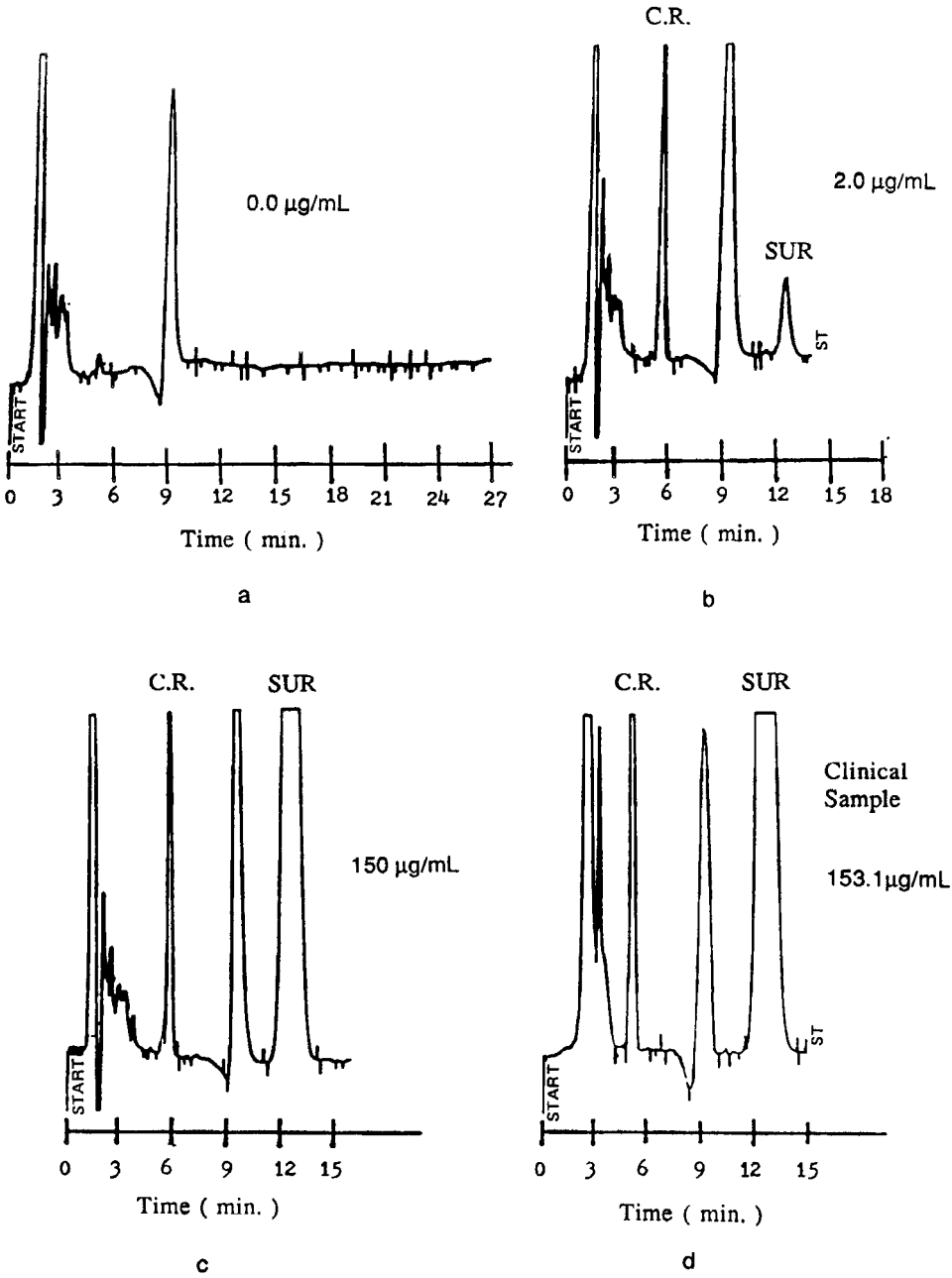

Figure 2: Typical chromatograms from the assay representing a) standard curve points of 0.0 µg/mL b) 2.0 µg/mL and c) 150 µg/mL. Figure 2d is a chromatogram of a typical clinical sample , this one corresponding to 153.1 µg/mL.

TABLE 1:LINEARITY

SURAMIN STANDARD CURVE
IN HUMAN PLASMA

µL SUR Spiked	Spiked Conc. (µg/mL)	Peak Height Ratio (SUR/CR)	Calc. Conc. (µg/mL)
0.00	0.00	0	-0.84
0.50	2.00	0.1080	1.93
1.25	5.00	0.2202	4.82
2.50	10.0	0.4616	11.0
5.0	20.0	0.7736	19.0
12.5	50.0	2.0011	50.6
17.5	70.0	2.8102	71.4
25.0	100.0	3.9088	99.6
37.5	150.0	5.8517	149.5

$y=0.0389x + 0.0328$, $r^2=0.9998$

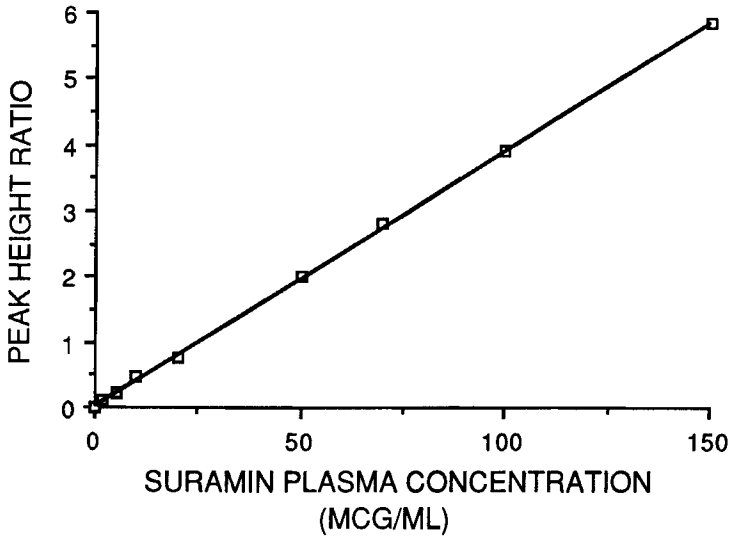

Figure 3: Graph of a standard curve showing the linearity of the assay.

of 0.9998, a slope of 0.0389, and a y-intercept of 0.0328 were obtained. Also included in Table 1 are the calculated concentrations of each point on the curve. All standard curves run in plasma yielded r^2 values of at least 0.999+, and the standard curves run in blank urine yielded curves with r^2 values of 0.9995+.

Precision

The inter-day precision of the method (Table 2) was run throughout an 86 day period with drug concentrations of 6, 20, and 100µg/mL. The coefficient of variation (CV) ranged from 2.76% to 4.62%.

Intra-day precision (Table 3), was run with drug concentrations of 5, 20, and 100µg/mL and had a CV range of 0.826% to 5.51%.

Recovery

Table 4 shows the percent recovery of SUR in plasma vs. H_2O. Spiked samples were prepared as described above except that CR was not added until just before N_2

TABLE 2:INTER-DAY PRECISION FOR SURAMIN IN PLASMA

DAY	HIGH CONCENTRATION (100µg/mL)	MEDIUM CONCENTRATION (20µg/mL)	LOW CONCENTRATION (6µg/mL)
1	91.44	19.58	5.66
2	91.64	19.40	5.42
9	95.00	19.84	5.79
15	92.96	18.72	5.51
16	99.33	19.57	5.81
85	103.8	20.12	5.49
86	95.40	20.39	6.07
MEAN	97.64	19.56	5.78
SD	4.51	0.539	0.229
%CV	4.62	2.76	3.96

TABLE 3: INTRA-DAY PRECISION FOR SURAMIN IN PLASMA

SAMPLE	HIGH CONCENTRATION (100µg/mL)	MEDIUM CONCENTRATION (20µg/mL)	LOW CONCENTRATION (5µg/mL)
1	97.64	20.30	5.84
2	104.3	20.49	5.09
3	99.14	19.94	5.17
4	99.07	20.15	5.02
5	100.7	20.38	5.11
6	101.2	20.23	4.99
7	98.94	20.13	5.10
8	98.59	20.26	4.86
MEAN	100.9	20.22	5.35
SD	2.09	0.167	0.295
%CV	2.07	0.826	5.51

TABLE 4: RECOVERY OF SURAMIN FROM HUMAN PLASMA

PEAK HEIGHT RATIO	HIGH CONCENTRATION (100µg/mL)	MEDIUM CONCENTRATION (20µg/mL)	LOW CONCENTRATION (5µg/mL)
Water	3.4102	0.6847	0.1716
Plasma	2.6662	0.5839	0.1418
Recovery (Plasma/ Water)	78.2%	85.3%	82.6%

evaporation. The peak height ratios of SUR/CR in plasma were determined as follows:

$$\%\text{Recovery} = \frac{(\text{peak height ratio of SUR/CR in plasma})}{(\text{peak height ratio of SUR/CR in water})} \times 100$$

The recovery for triple extractions ranged from 78.2% to 85.3%.

DISCUSSION AND CONCLUSION

In order to determine suramin levels in plasma and urine samples collected from AIDS patients being treated with this agent, we initially utilized the HPLC assay as described by Klecker, et. al.(4).We were, however, unable to reproduce the assay, and decided to develop a more simple assay using an isocratic mobile phase in place of a gradient system.

Trypan blue was used as the internal standard in the Klecker assay, but we observed that this agent had a shifting retention time and was associated with a number of small interfering peaks, believed to be due to impurities present in the trypan blue stock solution. Rather than purifying the trypan blue stock, congo red was used as our internal standard. Its chemical structure is similar to suramin, and the chromatographic peak shape is superior to that of trypan blue, having a retention time different from all endogenous plasma or interfering drug peaks. In addition, trypan blue is a suspected carcinogen whereas congo red is not.

Our sample preparation was similar to that used by the Klecker assay, with the exception of using TBACl to further enhance peak shape.

In our study, we were analyzing clinical samples with high drug concentrations and therefore less concerned with the detection of very low drug levels.In our assay quantification below 2μg/mL was poor, but no attempt was made to improve this sensitivity, since this level was sufficient for our purposes. Both the Klecker(4) and the Ruprecht(5) assays are quantifiable down to the 0.5μg/mL level, but only our assay and Ruprecht's have linearity through the 200μg/mL level.

In order to produce an accurate, simple assay that could be reproduced, two separate HPLC systems were set up using different types of pumps, detectors and injectors.

Different columns of the same manufacturer were also used and the assay was found to work equally well with both systems. This we felt was especially important since a wide variety of HPLC equipment is currently used. System II was used for all data recorded in this paper.

Two special procedures of note were required with this assay. First, the clinical samples assayed were taken from patients with AIDS, so special precautions were taken, including complete preparation of the samples under a P_2 biological hazard saftey fume hood, including autoclaving of liquid and solid infected wastes, wearing of gloves and protective gowns at all times, and decontamination of work surfaces and instruments.Deactivation of the AIDS virus was accomplished while preparing samples, by the addition of the the internal standard (in acetonitrile), (6). The samples, however, were still handled with great care as described above. Second, with the mobile phase at a relatively high pH of 6.8, the column life was greatly increased by washing weekly with filtered "nanopure" water for one hour at a flow rate of 1mL/min., followed by filtered HPLC grade methanol for one hour at the same rate.

In conclusion, we believe that this is a simple, accurate assay with great reproducability, for the detection of moderate to high levels of suramin in human plasma and urine. It requires neither unusual nor lengthy preparations and can be performed using different HPLC equipment.

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