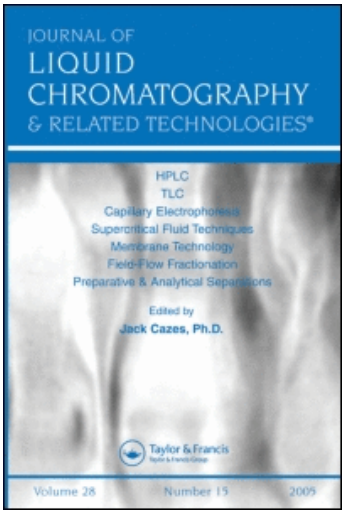

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A Rapid Isocratic HPLC Assay of Suramin (NSC 34936) in Human Plasma

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A RAPID ISOCRATIC HPLC ASSAY OF SURAMIN (NSC 34936) IN HUMAN PLASMA

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ABSTRACT

A reversed-phase, isocratic HPLC method was developed for the quantitation of suramin in plasma. The addition of acetonitrile containing the ion pairing agent tetrabutylammonium phosphate (0.05 M) and an internal standard, 2-naphthol, served to precipitate the protein of plasma and concurrently extract suramin into acetonitrile as the ion pair. After centrifugation, the supernatant was diluted with an equal volume of ammonium acetate buffer (0.01 M, pH 6.5) and an aliquot was injected on column. Chromatography was carried out on a 4 μm Nova-Pak C_{18} radial compression column (5 mm x 100 mm) using a water-methanol (51:49, v/v) mobile phase containing 0.01 M ammonium acetate buffer (pH 6.5) and 0.001 M tetrabutylammonium phosphate with detection by UV absorption at 238 nm. The run time for a single plasma sample was less than 10 min. Employing 25 μl of plasma, concentrations of suramin in the range 50-500 μg/ml were quantitated.

INTRODUCTION

Suramin is the hexasodium salt of a naphthyl urea compound with six aromatic sulfonic acid substituents [8,8'-(carbonylbis(imino-3,1-phenylene-carbonylimino(4-methyl-3,1-phenylene)carbonylimino))-bis-1,3,5-naphthalene-trisulfonic acid] which has been used as an antitrypanosomal agent for more than a half century (1). Identification of activity as a reverse transcriptase inhibitor with in vitro activity against human immunodeficiency virus (HTLV-III) stimulated its clinical evaluation in the treatment of

patients with the acquired immunodeficiency syndrome (AIDS) and AIDS related complex (ARC) (2-6). Adrenal insufficiency was found to be the dose-limiting toxicity during these clinical studies (7). Suramin has also been demonstrated to inhibit growth factor-related activities in the cell by preventing the binding of growth factors to their membrane receptors (8-12).

The initiation of clinical trials of suramin as an antitumor agent at the National Cancer Institute was prompted by its activity as a growth factor inhibitor (13) and also by its ability to cause adrenal necrosis. In cancer patients treated with an infusion of suramin, the plasma pharmacokinetics were reported to be variable (13). Plasma levels of suramin above 200 μg/ml correlated strongly with response in patients with cancers of the kidney, prostate, adrenal gland, and a variety of lymphomas (13). Neurologic toxicity, a major toxicity of suramin in patients, was associated with peak plasma levels greater than 300 μg/ml (13, 14). For peak suramin levels of 400 μg/ml or greater, the risk of significant neurotoxicity approached 100% (14).

The National Cancer Institute has very recently initiated phase II clinical trials of suramin in prostate cancer patients which will be undertaken at multiple institutions (15). The clinical studies require the monitoring of plasma levels during therapy. Infusion doses of the drug will be individualized to maintain plasma levels of suramin greater than 200 μg/ml and less than 300 μg/ml.

The plasma pharmacokinetics in AIDS patients treated with suramin (16) were monitored by an HPLC method in which suramin was isolated from plasma by a three-fold ion pair extraction with tetrabutylammonium phosphate (TBAP) into methanol (17). Chromatographic separation was achieved with a methanol gradient using a mobile phase containing TBAP and UV detection at 313 nm. Anticipating the need for monitoring plasma levels of suramin in the range 50-500 μg/ml in different laboratories by persons with varied experience in liquid chromatography, efforts were directed to enhance the efficiency of the extraction of suramin from plasma, to develop an isocratic chromatographic system, to reduce sample manipulation and the time required for the assay, and to use readily available equipment.

MATERIALS AND METHODS

Apparatus

Chromatography was performed using a model 114 pump (Beckman Instruments, Berkeley, CA), a WISP 712 automatic injector (Waters Associates, Milford, MA) and a

variable wavelength Spectroflow 783 programmable UV absorbance detector (ABI Analytical, Kratos Division, Ramsey, NJ) containing a 12 μl flow cell (path length 8 mm) was used. The 1 volt output of the detector was provided as the signal to a model 3393 recording integrator (Hewlett-Packard, Avondale, PA). The system was equipped with an RCM 8x10 cartridge holder containing a 5 mm x 100 mm Radial-Pak cartridge column packed with 4 μm Nova-Pak C_{18} stationary phase (Waters Associates). A Guard-Pak precolumn module fitted with a Nova-Pak C_{18} insert (Waters Associates) was installed before the analytical column and a 0.5 μm post-injector filter (Rainin Instrument Co., Woburn, MA) was employed.

UV spectra during chromatography were obtained with a second analytical system configured similarly but with a Hewlett-Packard (Palo Alto, CA) model 1040M diode array detector and a model 7125 manual injector (Rheodyne, Cotati, CA) equipped with a 100 μl loop.

A Cahn 25 electrobalance (Cahn Instruments, Inc., Ceritos, CA) was used for weighing milligram quantities of material during the preparation of stock solutions. All solutions were prepared in class A borosilicate glass volumetric flasks treated with a siliconizing reagent (Surfasil in toluene, Pierce Chemical Co., Rockford, IL). An Eppendorf model 5412 microcentrifuge (Brinkmann Instruments, Westbury, NY) was used for centrifuging assay samples contained in 1.5 ml polypropylene microcentrifuge tubes (VWR Scientific, San Francisco, CA).

Reagents and Chemicals

Suramin sodium (NSC 34936, lot XR781) was obtained from the Pharmaceutical Resources Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute and was used as the analytical reference standard in these studies. The internal standard, 2-naphthol, was purchased from the Eastman Kodak Co. (Rochester, NY). The methanol and acetonitrile were glass distilled (OmniSolv grade, E.M. Science, Cherry Hill, NJ). The water, double distilled and deionized, was filtered through a 0.2 μm Nylon-66 filter (Rainin Instrument Co., Woburn, MA) before use.

A 0.2 M TBAP solution was prepared by dissolving 6.8 g of HPLC grade tetrabutylammonium phosphate (Eastman Kodak) with water and diluting to 100 ml. Buffer solutions were prepared with analyzed reagent ammonium acetate (J.T. Baker Chemical Co., Phillipsburg, NJ) and analytical reagent glacial acetic acid (Mallinckrodt, Paris, KY). A 1.0 M ammonium acetate solution (pH 6.55) was made by combining 50 ml

of 2.0 M ammonium acetate (15.25 g NH_4Ac/100 ml) and 10 ml of 0.1 M acetic acid (608 µl glacial AcOH/100 ml) and diluting to 100 ml with water. Dilution of this solution 100-fold with water afforded a 0.01 M ammonium acetate buffer (pH 6.5).

Macromolecule Precipitant Solution

The internal standard stock solution was 1.0 mg/ml 2-naphthol in acetonitrile. A 500 µl aliquot of this solution was pipeted into a flask containing 50 ml of TBAP in acetonitrile (17.0 mg/ml), affording the solution used in the assay for precipitating plasma proteins and isolating the drug.

Plasma Standards

A 100.14 mg/ml aqueous stock solution of suramin was prepared. The initial plasma standard, containing approximately 500 µg/ml of the drug, was prepared by carefully diluting 125 µl of the stock solution to 25 ml with drug-free human plasma. Serial dilution of this standard with drug-free plasma afforded the range of concentrations employed for the standard curve.

Plasma Sample Preparation

In a microcentrifuge tube, 0.5 ml of acetonitrile containing the internal standard and TBAP (0.05 M) was added to 25 µl of plasma, effecting precipitation of plasma proteins. After thoroughly mixing for 0.5 min with a vortex action stirrer, the tubes were centrifuged at 12,000 x g for 10 min. A 250 µl aliquot of the resulting supernatant was added to an equal volume of 0.01 M ammonium acetate buffer (pH 6.5) in a second microcentrifuge tube, mixed by vortexing and transferred to a polypropylene automatic sampler insert. The injection volume was 10 µl.

Chromatographic Conditions

The mobile phase was prepared by mixing 10 ml of 1.0 M ammonium acetate buffer (pH 6.55), 5 ml of 0.2 M aqueous TBAP solution and 495 ml of water with 490 ml of methanol. This solution was degassed by placing the flask in a ultrasonic bath for 15 min prior to chromatography. Before using the Guard-Pak precolumn inserts, which were replaced on a daily basis, they were wetted with methanol and then equilibrated with the mobile phase for 20 min.

Chromatography was performed at ambient temperature with a flow rate of 1.0 ml/min. Absorbance of the column effluent was monitored at a wavelength of 238 nm. The integrator was configured to report peak areas employing a 0.2 min peak width, a threshold setting of 1, and baseline construction through each detected valley point. A 0.2 cm/min chart speed with an attenuation of 3 were used for plotting the chromatograms.

Quantitation

Standard curves were run on a daily basis. The peak area ratios of suramin to the internal standard were plotted as a function of suramin concentration. The best fit straight line was determined by unweighted linear least squares regression, without inclusion of the origin, to calculate the slope, y-intercept and correlation coefficient. The concentration of suramin, expressed as μg/ml in terms of its hexasodium salt, in unknown samples was calculated using the results of the regression analysis.

Recovery as a Function of Ion Pairing Agent Concentration

The concentration of TBAP in the acetonitrile precipitant solution was varied in order to establish the amount of ion pairing agent required for optimal recovery of suramin from a plasma sample with a single 500 μl addition of this solution. Six 25 μl aliquots of the 500 μg/ml plasma standard were individually prepared for chromatography at each concentration of TBAP employed. The average suramin peak area and standard deviation for each set of replicates were determined.

Absolute Recovery

A series of suramin sodium standard solutions in water containing the same concentrations of drug as the plasma standards was prepared. The aqueous standards were prepared for chromatography in the same manner as the plasma standards. Six replicate assays of both standard sets were performed at suramin concentrations ranging from approximately 50 to 500 μg/ml. The samples were chromatographed in pairs such that each plasma standard immediately preceded an aqueous standard of the same concentration. Accordingly, ratios of the suramin peak area for the precipitated plasma standard to that of the paired aqueous sample provided a measure of the absolute recovery. Absolute recovery of the internal standard was determined by a similar procedure employing a single concentration.

RESULTS

Plasma Sample Preparation

The protein of plasma samples containing suramin was precipitated with acetonitrile containing TBAP and the internal standard. After dilution of the supernatant with an equal volume of ammonium acetate buffer (0.01 M, pH 6.5) to adjust the solvent strength, the sample was chromatographed using a reversed-phase system containing TBAP with detection at 238 nm. The duration of chromatography for a single sample was 10 min, permitting completion of sample preparation and chromatography of a calibration curve, consisting of 10 plasma standards plus a drug-free sample, in approximately two hours.

Extraction Efficiency

The recovery of suramin from the 500 μg/ml plasma standard as a function of TBAP concentration in the acetonitrile solution employed for the simultaneous precipitation of plasma protein and extraction of the drug is shown in Figure 1. Recovery of the drug from 25 μl of plasma increased asymptotically with the amount of TBAP present in 500 μl of acetonitrile, becoming invariant from a practical consideration when the amount of added ion pairing agent exceeded 20 μmoles. Accordingly, as indicated in Table 1, the absolute recovery of drug from plasma standards containing 50-500 μg/ml of suramin employing a 0.05 M TBAP acetonitrile solution was essentially quantitative. The internal standard absolute recovery was found to be 99.54 ± 0.02% (n = 36) under these conditions.

Chromatography of Suramin

Figure 2A shows a chromatogram of drug and internal standard-free human plasma using 238 nm detection. The plasma samples generally afforded clean chromatograms with no endogenous peaks which interfered with the drug or internal standard. Chromatograms determined with a variable wavelength detector at 238 nm of human plasma spiked with suramin having concentrations of 50.07, 250.35 and 500.70 μg/ml are shown in Figures 2B, 2C, and 2D. Suramin eluted prior to the internal standard, 2-naphthol, under the chromatographic conditions which were employed. Typical

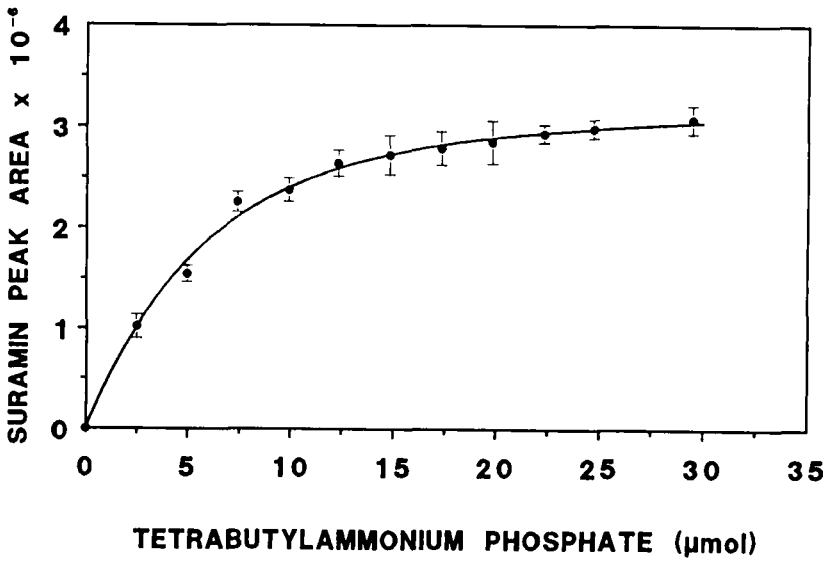

FIGURE 1. Recovery of drug from 25 μl of plasma containing 482.05 μg/ml suramin with 500 μl of tetrabutyammonium phosphate solution in acetonitrile. Experimental values are plotted with two standard deviation error bars.

TABLE 1

Absolute Recovery of Suramin from Human Plasma[a]

Amount added (μg/ml)	Mean recovery %	Coefficient of variation %
50.07	101.95	2.44
100.14	102.46	3.29
200.28	99.30	2.49
300.42	103.22	2.83
400.56	101.59	4.27
500.70	100.52	5.32

[a] Number of replicates = 6.

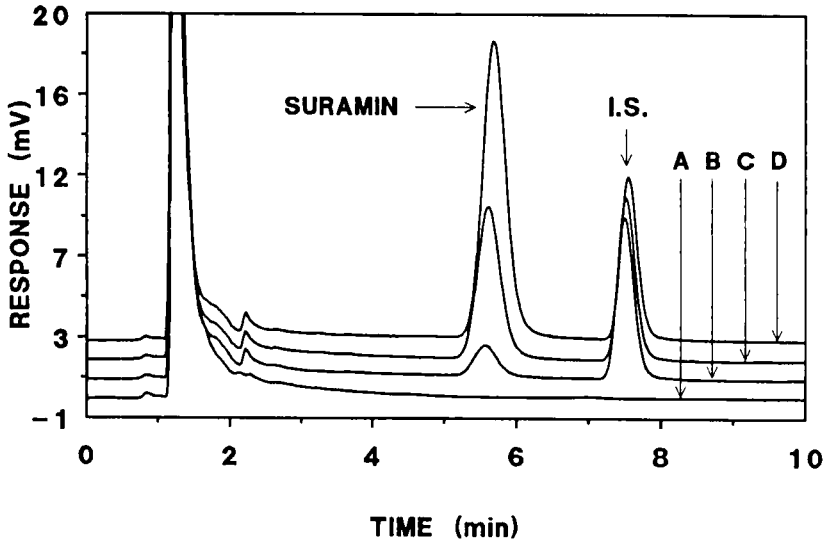

FIGURE 2. Representative liquid chromatograms of human plasma standard curve samples with detection at 238 nm. (A) drug and internal standard-free plasma, and plasma spiked with suramin to provide concentrations of (B) 50.07 μg/ml, (C) 250.35 μg/ml and (D) 500.70 μg/ml. The baseline of chromatograms (B), (C) and (D) were incrementally offset from zero by 1 mV.

retention times (mean ± standard deviation), acquired from a series of plasma samples chromatographed on a single day, were 5.62 ± 0.04 min for the drug (n=10) and 7.53 ± 0.02 min for the internal standard (n=10).

The retention time for each component was extremely consistent on any given day. However, it was observed that the retention of suramin was significantly more sensitive toward slight variations in the methanol content of the mobile phase, temperature fluctuations and the precolumn insert replacements than 2-naphthol. Accordingly, the fraction of methanol in the mobile phase was adjusted as necessary, typically by no more than ± 0.5%, such that the drug peak eluted within 5.5-6.0 min. This provided sufficient separation of the suramin peak from both the solvent front and internal standard for accurate peak area determinations.

Calibration Curves

Figure 3 represents a typical calibration curve in which the ratio of the peak area of suramin to that of the internal standard is directly proportional to the suramin

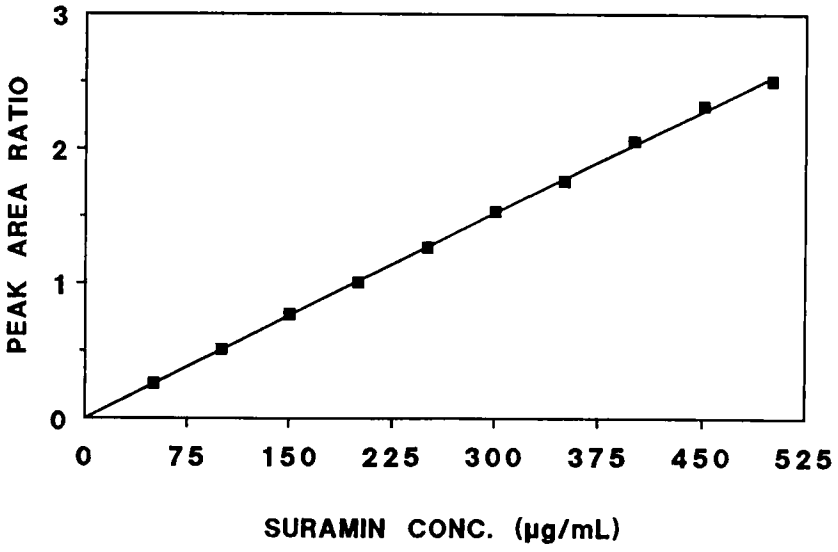

FIGURE 3. Typical standard curve of peak area ratio (suramin/internal standard) vs. concentration for 50-500 µg/ml of suramin in human plasma: slope, 0.005072; y-intercept, 0.001120; correlation coefficient, 0.9997.

concentration. The best fit lines of the calibration curves having a 10-fold range of concentrations were obtained by linear regression analysis without the use of a weighting factor. The correlation coefficients were generally greater than 0.999.

Assay Reproducibility

Plasma standards were assayed on 3 days during a 2 week period from a single stock solution of suramin. The relative recovery of suramin from plasma and reproducibility of the assay for the range of concentrations of the standard curve determined during this period are summarized in Table 2. The coefficients of variation for eight replicate assays of each plasma standard, which ranged from 0.83 to 3.11%, showed that the analytical method is reproducible. Relative recoveries ranged from 98.75 to 101.13% of the amount of added suramin. The coefficient of variation for the slope of the linear regression curves (n=8) was 2.78%, demonstrating that the assay was consistent during this period.

TABLE 2

Relative Recovery and Reproduciblity of the Analytical Method for Suramin in Human Plasma[a]

Amount added (μg/ml)	Mean amount found (μg/ml)	Coefficient of variation %	Recovery %
50.07	49.57	3.11	99.00
100.14	98.93	1.49	98.79
150.21	149.54	1.21	99.55
200.28	199.15	1.60	99.44
250.35	250.82	0.83	100.19
300.42	302.83	1.99	100.80
350.49	352.33	0.93	100.53
400.56	405.09	0.84	101.13
450.63	449.68	1.48	99.79
500.70	494.46	1.37	98.75

[a] Number of replicates = 8.

Specificity of the Analytical Method

Studies to establish that endogenous plasma constituents do not interfere with the analytical method were carried out with a similar isocratic system, column and mobile phase but using a diode array detector to permit the acquisition of UV spectra during the elution of the suramin and internal standard chromatographic peaks. An injection volume of 100 μl was employed. Three-dimensional chromatograms for a drug-free plasma sample without internal standard and the 250 μg/ml standard, the latter showing peaks for suramin and internal standard, are shown in Figure 4. The presence of metabolites in the plasma of patients treated with suramin has not been reported (17).

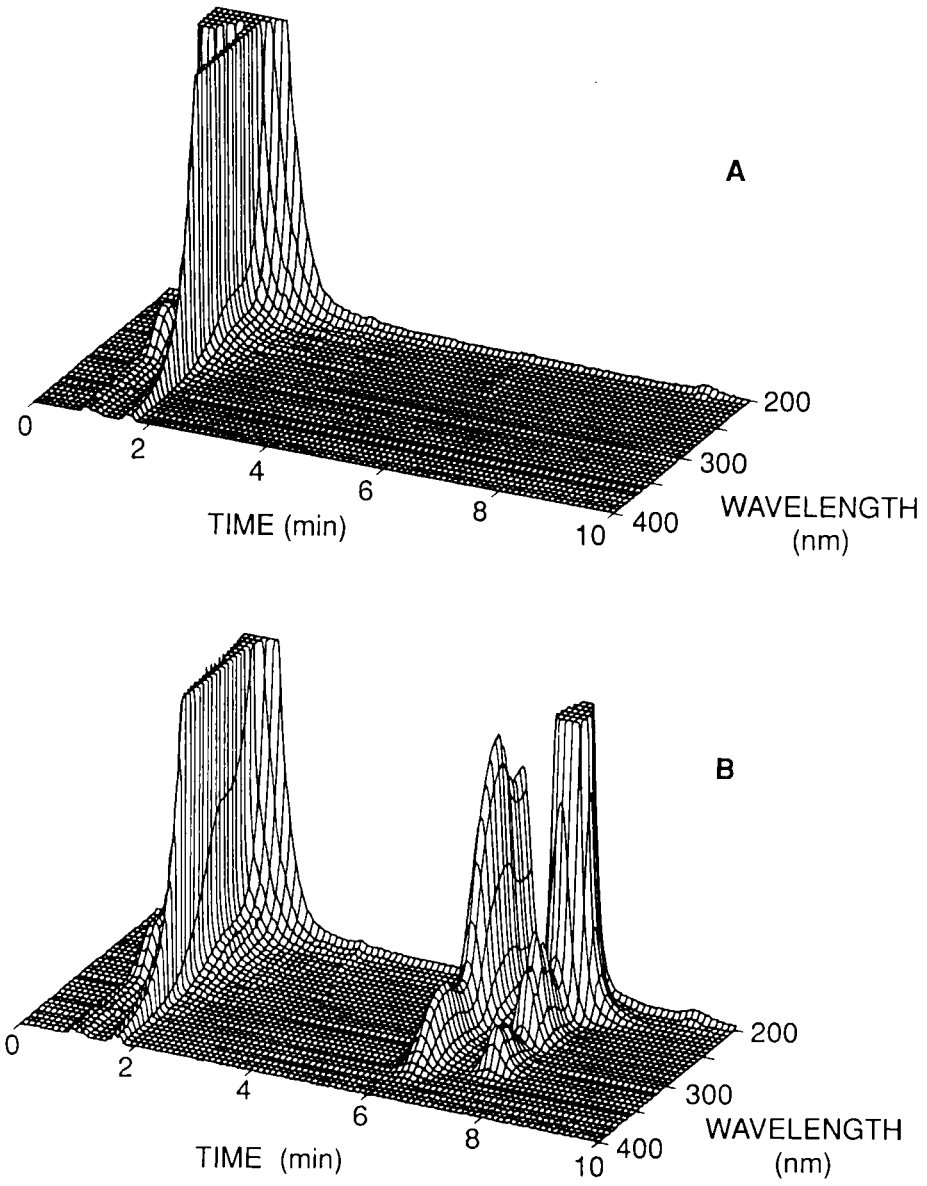

FIGURE 4. Three-dimensional chromatograms truncated at 65 mAU of (A) drug and internal standard-free human plasma and (B) 250.35 μg/ml suramin in plasma with internal standard, demonstrating that the extract contains no endogenous plasma constituents interfering with the detection of suramin (6.2 min) or the internal standard (7.6 min).

DISCUSSION

Suramin sodium is a highly water soluble compound which exhibits appreciable UV absorption, with λ_{max} at 238 and 313 nm in aqueous solution (Figure 5A). However, the presence of six aromatic sulfonate functional groups renders the molecule relatively insoluble in nonpolar organic solvents. The drug also exhibits poor solubility in the more polar water miscible solvents methanol and acetonitrile, which are commonly used in the preparation of biological samples for liquid chromatography.

The solubilization of suramin in methanol by ion pair formation with tetrabutylammonium phosphate (TBAP) was utilized in the analytical method developed by Klecker and Collins (17). A single extraction of the volume of plasma taken for assay (0.5 ml) with methanol (1 ml) after the addition of 100 μl of 0.5 M TBAP afforded 67% recovery of the drug. This method required three extractions in order to achieve an extraction efficiency of 97%. Trypan blue was employed as an internal standard. Chromatographic separation was achieved with a methanol gradient using a mobile phase containing TBAP and UV detection was at 313 nm. The method has been employed recently at the National Cancer Institute for measurement of plasma levels in cancer patients treated with suramin (13).

In order to facilitate the general use of an analytical method for quantitating plasma levels of suramin in cancer patients, studies were undertaken to enhance the ion pair extraction and to utilize isocratic chromatographic separation. Accordingly, the isolation of suramin from plasma by ion pair extraction was studied. It was found that virtually quantitative recovery of drug with acetonitrile in the presence of TBAP could be achieved with a single addition of the reagent for the concentration range of interest (Table 1). Variation with respect to the concentration of ion pairing agent in the solvent demonstrated that optimal extraction efficiency from a 25 μl plasma sample was effected with 500 μl of 0.05 M TBAP in acetonitrile (Figure 1).

Not only could extraction be achieved in a single step, but also a great deal of the endogenous polar plasma constituents which are readily extracted by methanol do not extract into acetonitrile. Interferences in the chromatograms after extraction with acetonitrile containing 0.05 M TBAP were significantly lower than following the triple extraction with methanol subsequent to the addition of TBAP. This reduction in the extraction of plasma constituents permitted detection at either the 238 nm or 313 nm absorption maximum (Figure 5A). Inasmuch as the absorptivity of suramin at 238 nm (ϵ 114,000 $M^{-1}cm^{-1}$) is significantly greater than at 313 nm (ϵ 27,100 $M^{-1}cm^{-1}$), the assay for the concentration range studied required only 25 μl of plasma.

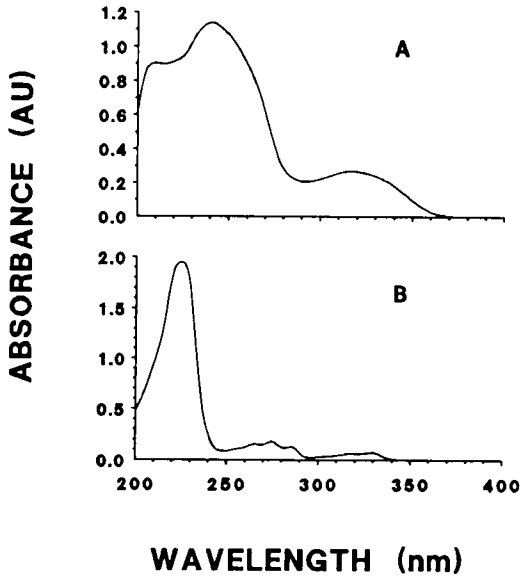

FIGURE 5. UV absorption spectra of solutions in the analytical mobile phase of (A) 10 μM suramin and (B) 50 μM 2-naphthol, the internal standard, referenced to mobile phase.

The concentration range of the calibration curves (50-500 μg/ml) was based upon the plasma level monitoring required for the clinical trials. It was determined that the lower limit of the standard curve could be extended to 5 μg/ml without changing the injection volume. Replicate assays (n=6) of a 5.0 μg/ml sample of suramin in plasma gave a 6.97% coefficient of variation. Preliminary studies indicated that, with minor modifications of the mobile phase to increase the drug and internal standard retention times, significantly lower suramin levels may be quantitated by employing a larger injection volume and 50-100 μl of plasma.

In the present method, the standard curves were linear for the entire concentration range studied demonstrating that for this range, the extraction efficiency was constant. Calibration curves in the gradient method for suramin in plasma were reported as plots of the peak height ratio (PHR; suramin/internal standard) against the concentration of suramin (0.5-300 μg/ml) using $1/(PHR)^2$ as a weighting factor (17). There was good linearity from 0.5 to 100 μg/ml. However, the 200 μg/ml plasma standard showed a small negative deviation from the regression curve and the negative deviation of the 300

μg/ml standard was substantially greater. The departure from linearity is very likely due to decreased extraction efficiency at the higher concentrations.

The isocratic mobile phase containing the ion pairing agent used in the present work was derived from the method of Klecker and Collins (17). An internal standard (2-naphthol) was included solely to monitor pipetting and reproducibility. Selection of this compound considered primarily its chromatographic retention and UV absorption (Figure 5B) properties. In the earlier method (17), trypan blue, which was employed as the internal standard, served to monitor extraction recovery. The authors noted that commercial samples of trypan blue vary in purity and may contain a contaminant which co-elutes with suramin. Furthermore, the absorptivity of trypan blue at 313 nm is significantly less than that of suramin. In order to achieve the peak height ratios of the reported calibration curve, a very high concentration of trypan blue relative to suramin would have been required. This may have contributed to the observed incomplete recovery at higher drug levels with a single extraction according to their procedure.

In summary, a facile analytical method for suramin in plasma was developed to permit drug level monitoring in cancer patients. The method is derived from that previously reported (17) and necessitates minimal sample manipulation. Quantitation of plasma levels in the range 50-500 μg/ml requires only 25 μl of plasma.

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