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# Development of a high-performance liquid chromatographic method for the analysis of staurosporine

L.R. Gurley*, K.O. Umbarger, J.M. Kim, E.M. Bradbury, B.E. Lehnert

*Life Sciences Division, Mail Stop M-880, Los Alamos National Laboratory, Los Alamos, NM 87545, USA*

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**Abstract**

Staurosporine (Stsp), a protein kinase inhibitor, has been found to have a differential effect on the proliferation of normal and transformed cells in vitro. Hence, Stsp might be used in cancer therapy to arrest normal proliferating cells in G1, while permitting tumor cells to continue proliferation. The patient could then be treated with a therapeutic agent of maximum toxicity for actively proliferating tumor cells. To facilitate investigations of Stsp in vivo, we have developed an HPLC method for measuring the levels of Stsp in blood. Using a rat model, plasma containing Stsp is treated with acetone to precipitate proteins and extract the Stsp. The acetone extract is then subjected to reversed-phase HPLC on a μBondapak $C_{18}$ column. Using a linear elution gradient of acetonitrile containing trifluoroacetic acid, Stsp elutes as a sharp peak at ca. 35 min which can be detected by UV absorption at 292 nm. No blood or reagent components interfere with its quantification. The calibration curve, ranging from 0.1 to 2.0 μg Stsp, demonstrated a linear response to Stsp concentration having a correlation coefficient ($r^2$) of 0.990. Precision analysis demonstrated that the method will yield results that are ±11.6% from the mean 95% (two standard deviations) of the time. This method was used to measure Stsp levels in plasma after administering an injection of 0.2 mg Stsp into the jugular vein of rats. No Stsp could be detected in the plasma 5 min after injection, even though enough Stsp was administered to be easily detectable if it was simply contained in the plasma. Thus, it is concluded that some compartment other than the plasma must adsorb the Stsp from the plasma and sequester it in vivo.

## 1. Introduction

The regulation of mammalian cell proliferation has long been thought to involve kinase-mediated mechanisms of protein phosphorylation [1–12]. In our laboratory, recent investigations by Crissman et al. [13] were conducted to determine the effects of the general protein kinase inhibitor staurosporine (Stsp) on the proliferation of cultured mammalian cells. That work demonstrated that Stsp has a differential effect on the cell cycle progression of normal and transformed cells as they pass through G1 and S phase. It was found that low levels of Stsp (10 ng/ml) reversibly arrested normal cells in early G1 phase while much higher concentrations of Stsp (50 ng/ml) had no effect on G1 progression and DNA replication in transformed tumor cells. Those studies had major implications for the development of new strategies in cancer diagnosis and therapy [13].

One of the most interesting of those strategies

* Corresponding author.

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involved using this drug's capacity to arrest normal cells, but not transformed cells, in G1. According to this strategy, a cancer patient would first be treated with low levels of Stsp which would temporarily arrest the proliferation of normal cells in G1. The patient could then be treated with a therapeutic agent of maximum toxicity for proliferating tumor cells. The normally proliferating cells of tissues such as bone marrow and intestinal mucosa, which are usually killed by such therapeutic agents, would be spared due to their lack of DNA synthesis while the tumor cells would continue to progress through S phase and be killed. This strategy would allow a far more effective therapeutic dose of tumor-killing agent to be delivered to the proliferating tumor cells while reducing the harmful side-effects on the normal tissues whose cell proliferation was temporarily inhibited by Stsp and thus insensitive to the toxic therapeutic agent [13].

To determine the feasibility of this strategy, Stsp was administered to rats to produce G1 arrest in its normally proliferating tissues. Flow cytometry analysis of bone marrow cells indicated that G1 arrest was not accomplished by this treatment [14]. This suggested that the Stsp was not reaching its target tissues in vivo. From these preliminary studies, it was clear that it would be necessary to assay the level of Stsp in the blood in order to determine the dose levels and the time schedules for administering the drug. This paper reports the development of an analysis procedure for Stsp in blood samples using HPLC.

## 2. Experimental

### *2.1. Staurosporine*

Stsp was obtained from Kamiya (Thousand Oaks, CA, USA). For spectrophotometry and initial chromatography experiments, Stsp was dissolved in dimethyl sulfoxide (DMSO) (J.T. Baker, Phillipsburg, NJ, USA) at a concentration of 0.5 $\mu$g/ml. This stock solution was then diluted to appropriate concentrations in various solvents, serum, or plasma for individual experiments. In the final stages of the development of the Stsp assay and in the biological experiments that followed, Stsp was dissolved in polyethylene glycol-400 (PEG) (J.T. Baker) at a concentration of 1 $\mu$g/$\mu$l. This stock solution was then diluted to appropriate concentrations in various solvents, plasma or whole blood samples.

### *2.2. Spectrophotometry*

The absorption spectrum of Stsp solutions was obtained using a Gilford Model 250 spectrophotometer equipped with a Model 2430 wavelength scanner and a Model 2451 curette positioner fitted with a single dual-compartment cuvette containing sample on one side and blank solution on the other. This single beam instrument alternately measured the absorption of the sample and blank using the reciprocating motion of the curette carriage while scanning the wavelengths. The blank was subtracted from the sample absorption electronically to produce the absorption spectrum.

### *2.3. Animals, blood collection, and blood processing*

Adult, male Fisher 344 rats, specific-pathogen-free were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA). Whole blood to be used for serum and plasma preparations was collected from rats anesthetized with an i.p. injection of 50 mg sodium pentobarbital. The blood was removed by cardiac puncture, using an unheparinized 10 ml syringe. Generally, 5–7 ml blood was collected per rat. To prepare serum, the blood was placed in an unheparinized centrifuge tube and was allowed to stand 30 min at room temperature to clot. The sample was then centrifuged at 800 *g* in a clinical centrifuge for 20 min and the supernatant serum fluid was removed.

To prepare plasma, the blood was placed immediately in a 7-ml draw, sterile blood collection tube (Vacutainer) containing either 100 USP units of sodium heparin or 10.5 mg of disodium EDTA (Bacon, Dickinson, Rutherford, NJ, USA). The blood was then centrifuged at 800 *g* in a clinical centrifuge for 6.5 min. The superna-

tant plasma fluid was removed with a positive displacement 250-μl micropipetter (Rainin, Emoryville, CA, USA) and placed in a separate tube. The hematocrit of rat blood at our altitude is ca. 50%. Thus, the volume of plasma and red blood cells (RBC) was approximately equal.

To inject Stsp into these rats, the animals were anesthetized using enfluorane in air (5% v/v, 2 l/min) and a sterile catheter filled with a 3-cm silastic tubing tip was inserted into the right jugular vein and advanced to place the tip just above the right atrium of the heart. The tubing was pre-filled with a 10% heparinized saline solution to prevent clotting at the tip. The catheter was attached to a 1-ml syringe and a single bolus injection of 0.2 mg Stsp dissolved in a PEG–10% aqueous glucose (1:1) solution was administered. A chase solution of 0.5 ml 10% aqueous glucose was then injected to clear the catheter dead space (0.2 ml). At 5 or 15 min after injection of Stsp, blood was collected from the rats by withdrawing it from just above the right atrium using the i.v. catheter inserted in the jugular vein. To prevent clotting, a 10-ml syringe prerinsed with heparin solution was used. The blood was immediately placed in a blood collection tube containing disodium EDTA. Plasma was obtained from these samples as described above.

### 2.4. High-performance liquid chromatography

Reversed-phase HPLC was performed on samples using a μBondapak $C_{18}$ Radial-PAK flexible walled cartridge (10 cm × 8 mm I.D.) (Waters, Milford, MA, USA). This cartridge was contained in a Waters Z-Module, which radially compressed the cartridge to produce a uniformly dense, stable column free of void pockets and wall effects [15]. The water and acetonitrile used in the HPLC system were HPLC grade (J.T. Baker) and the trifluoroacetic acid (TFA) was spectrograde (Pierce Chemical, Rockford, IL, USA).

The HPLC instrument consisted of three Waters Model 6000A solvent delivery pumps attached in series. Pump A was the closest to the column and pump C was the most distant. Pump C delivered water–0.2% TFA, pump B delivered 50% water–50% $CH_3CN$–0.2% TFA and pump A delivered $CH_3CN$–0.2% TFA. Control of the pump flow rates and the solvent gradients was achieved using a Digital Model 350 computer interfaced with the pumps by a Waters Model 840 system interface module. The UV absorption of the column effluent was monitored at four different wavelengths using a Waters Model 490 multiwavelength flow detector.

Before each HPLC run, the column was equilibrated for 20 min with water–0.2% TFA (aqueous TFA) at a flow-rate of 1 ml/min. Samples whose volumes were less than 2 ml were loaded on the column by injection using a Waters U6K injector. Samples greater than 2 ml were loaded by dipping the intake line of the pump into the sample tube and pumping the sample through the column at 1 ml/min. To ensure quantitative transfer of all the sample to the column, 5 ml aqueous TFA was added to the empty sample tube and it was pumped through the column to rinse the tube, pump, and pump lines. Following this loading, the pumping of aqueous TFA was continued until the column effluent absorbancy at 292 nm was less than 1.

Stsp was eluted from the column with a linear gradient of acetonitrile in aqueous TFA that progressed from water–0.2% TFA to $CH_3CN$–0.2% TFA in 60 min at a flow-rate of 1 ml/min. This was achieved by programming a linear gradient running from 100% C to 100% B in 30 min and 100% B to 100% A in 30 min. This pump arrangement eliminated the mixing of water–TFA directly with $CH_3CN$–TFA which produces baseline oscillation artifacts [16]. At the end of each run, the column was returned to the initial solvent conditions using a rapid 10-min linear gradient. The column was then equilibrated for the next run with aqueous TFA for 20 min.

### 2.5. Standard procedure developed for staurosporine analysis

Blood was collected in tubes containing disodium EDTA. For in vitro experiments, Stsp dissolved in PEG was added and the whole blood was incubated 15 min in a 37°C water bath. The blood was then centrifuged at room temperature

and the plasma was removed from the RBC as described above. Using a positive displacement 250-$\mu$l micropipetter, 0.1 ml of either plasma or RBC was placed in a 12-ml glass centrifuge tube and 1 ml acetone was added which precipitated the proteins immediately. The mixture was stirred with a glass rod and centrifuged at 800 *g* for 5 min. The supernatant acetone containing the Stsp was withdrawn and placed in another tube. The precipitate was washed twice with 0.5 ml acetone recovering the acetone each time the same way and adding it to the original extract. The acetone extract was diluted with 4.5 ml aqueous TFA. This 6.6 ml sample was then pumped through the HPLC at 1 ml/min followed by a 5 ml aqueous TFA tube rinse. The column was flushed isocratically with aqueous TFA for another 12 min to pump out the acetone. Stsp was then eluted with a 60-min linear gradient of acetonitrile as described above. The Stsp peak detected at 292 nm was recorded in the computer. The area of this peak was quantified using the Data Process program of the Waters 840 Data and Chromatography Control Station. The area of the peak was then converted to $\mu$g Stsp using a standard curve.

## 3. Results

### *3.1. Spectrophotometry of staurosporine*

The first objective of this project was to develop a quick simple method for measuring Stsp in rat blood. Staurosporine is a microbial alkaloid [17] and potent protein kinase inhibitor [18,19] having the structure shown in Fig. 1A. Because of resonance within its structure, Stsp was known to exhibit a characteristic UV absorption spectra [17] that might be useful for measuring its presence in biological samples. Therefore, spectrophotometric analysis was explored as a method of detection. When Stsp was dissolved in DMSO, it was found to have a strong absorbance peak at 296 nm and a weaker peak at 335 nm (Fig. 1A). However, when Stsp was dissolved in rat serum at a concentration of 17.5 $\mu$g/ml, the absorbancy of serum at 296 nm was so great that the Stsp could not be detected above the high background. The absorbancy of Stsp could be detected above that of serum at 335 nm but the background absorbance was considered to be too high for sentitive detection of the low concentrations of Stsp expected in our in vivo experiments (5 ng/ml to 20 $\mu$g/ml).

### *3.2. HPLC of Stsp in plasma*

The above experiments indicated that simple direct measurements of Stsp in blood samples was not possible and that the following things would have to be accomplished in order to analyze Stsp in blood samples: (1) The Stsp would have to be separated from the plasma proteins that interfere with detection. (2) The Stsp would have to be separated from the soluble low molecular mass plasma constituents that interfere with detection. (3) The Stsp would have to be separated from the reagents used to solubilize the Stsp (DMSO, ethanol, acetone, etc.) and from the reagents used to deproteinize the plasma which might interfere with detection. (4) The Stsp would have to be concentrated into a small volume to raise its concentration to detectable levels.

From our experience, we have found that reversed-phase HPLC is well suited for these tasks [20–25]. Therefore, we initiated experiments to find appropriate HPLC conditions to accomplish the analysis of Stsp using this technology. An elution solvent commonly used for reversed-phase HPLC is acetonitrile containing trifluoroacetic acid (TFA). To determine if Stsp could be detected in this solvent, the absorption spectrum of 5 $\mu$g Stsp/10 $\mu$l DMSO dissolved in 92% acetonitrile containing 0.2% TFA was measured (Fig. 1B). The high absorption below 250 nm was found to be due to DMSO. However, the background absorption was very low at 296 nm, the expected absorption peak of Stsp (Fig. 1A). When Stsp was added to this acetonrile–TFA solvent at a concentration of 5 $\mu$g/ml, the Stsp was detected with good sensitivity with a maximum absorbancy downshifted slightly to 292 nm (Fig. 1B). These results are similar to those observed for Stsp in methanol [17].

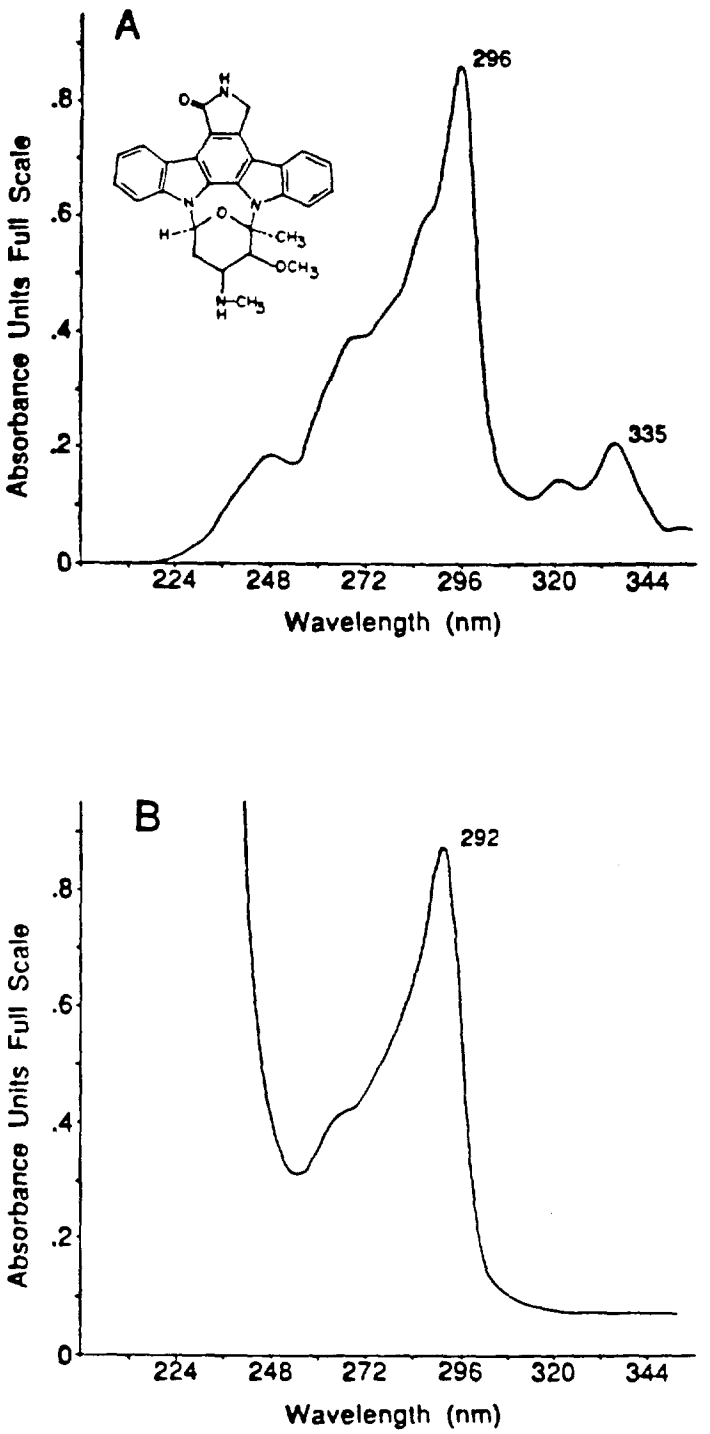


Fig. 1. Spectrophotometry of staurosporine. (A) Structure of Stsp (insert) and absorbance spectrum of 5 μg Stsp/ml DMSO using DMSO as a blank. (B) Absorbance of 5 μg Stsp/10 μl DMSO in 92% acetonitrile–0.2% TFA using 92% acetonitrile–0.2% TFA as a blank.

To determine the elution conditions of Stsp from a reversed-phase HPLC column, 5 μg Stsp dissolved in 10 μl DMSO was injected into a μBondapak $C_{18}$ column equilibrated with water containing 0.2% TFA. Elution of the Stsp was accomplished with a linear gradient of acetonitrile in aqueous 0.2% TFA that increased from 0 to 100% acetonitrile in 60 min. Using a multi-wavelength UV monitor, Stsp was detected eluting at 36 min or approximately 60% acetonitrile (Fig. 2A). As expected from the spectral analysis in Fig. 1, detection of Stsp was about 25% better at 292 nm (Fig. 2A) than at the 280 nm wavelength usually used for protein detection. Detection of Stsp at 215 nm, another wavelength frequently used for protein detection [21] was only one-third that at 292 nm. DMSO, in which the Stsp was dissolved, was eluted in the first 10 min and was only detected at 215 nm.

To determine if Stsp could be fractionated from plasma proteins by this HPLC method, 1 ml of whole plasma was injected into the system. The bulk of the plasma components eluted between 30 and 45 min (Fig. 2B). Detection of the plasma proteins was greatest at 215 nm and much less at 280 nm. While protein detection at 292 nm (Fig. 2B) was only one-third that at 280 nm, the elution of these proteins coincided with that of Stsp (Fig. 2A). From these experiments, it was concluded that Stsp could be detected by UV absorption in HPLC effluents, but it would have to be separated from the plasma proteins before it could be reliably quantified.

### 3.3. HPLC of Stsp in acetone extracts

Since plasma constituents interfered with the detection of Stsp, we decided to remove the plasma proteins and see if the Stsp could then be detected in the deproteinized fluid. In previous work, we had found that proteins could be effectively removed from plasma by precipitation with 10 volumes of acetone [20]. Therefore a solution of 25 μg Stsp dissolved in 50 μl of DMSO (0.5 μg/μl) was added to 100 μl plasma and 1000 μl acetone was added to precipitate the plasma proteins. The proteins were removed by centrifugation and the supernatant acetone was

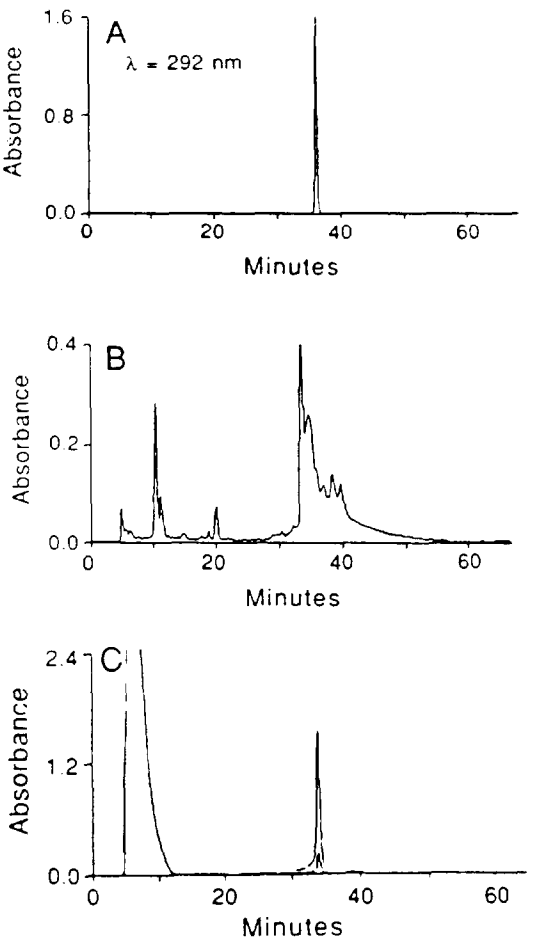


Fig. 2. Comparison of the UV absorption profiles of staurosporine and plasma proteins eluted from an HPLC $C_{18}$ reversed-phase column with a linear gradient of acetonitrile in aqueous 0.2% TFA. (A) A solution of 5 μg Stsp in 10 μl DMSO was injected into the HPLC and the eluted components were detected at 292 nm. (B) A 1-ml sample of whole plasma was injected into the HPLC and the eluted components were detected at 292 nm. (C) The proteins in a plasma sample containing 25 μg Stsp and 4.5% DMSO were removed by acetone precipitation and the acetone extract was injected into the HPLC. The eluted components were detected at 292 nm (large peak at 35 min). The precipitated proteins were washed twice with acetone and the acetone wash was injected into the HPLC. The eluted components were detected at 292 nm (small peak at 35 min).

injected into the HPLC column. The Stsp was eluted after 30 min (Fig. 2C). No other substance was detected at 292 nm except a large amount of material eluting in the first 10 min. All the

acetone, DMSO, and acetone-soluble plasma constituents were eluted in this initial peak (Fig. 2C). Monitoring the chromatography of the plasma acetone extract at 215 nm demonstrated that no proteins were eluted 30–45 min in the chromatogram, i.e., the acetone treatment had precipitated all the plasma proteins and none were solubilized to interfere with Stsp elution or detection after 30 min.

The acetone precipitated proteins were washed twice with 500 μl acetone and this 1000 μl of acetone wash was injected into the HPLC. A small amount of Stsp was detected amounting to about 12% of the amount initially extracted (small peak in Fig. 2C). Thus, washing the protein precipitate and adding the wash to the initial extract is necessary for a quantitative recovery.

### 3.4. Recovery of Stsp from acetone

While it was apparent that Stsp could be detected in the acetone extracts of plasma (Fig. 2C), there was concern that the Stsp might not be quantitatively adsorbed on the reversed-phase $C_{18}$ column in the presence of an organic solvent such as acetone. To determine if such solvent solubility losses did occur, 25 μg Stsp dissolved in 50 μl of DMSO was added to 1 ml acetone and subjected to HPLC (Fig. 3A). When this was compared to the HPLC of 25 μg Stsp in DMSO added to 11 ml of 0.2% aqueous TFA (Fig. 3B), it was found that only half of the Stsp was recovered from the acetone sample, even though the volume of the acetone sample was much smaller than that of the aqueous sample. Thus, it appeared that the acetone was not sufficiently polar to promote efficient adsorption of Stsp on the $C_{18}$ column.

In the past, we have found that such solubility losses can be eliminated by simply diluting the organic solvent with water to the point that the polarity of the sample solvent is increased sufficiently to promote total adsorption of the analyte to the column [21]. When the 1-ml acetone sample in Fig. 3A was added to 10 ml aqueous 0.2% TFA and this 1:10 diluted sample was subjected to HPLC, all the Stsp was recovered

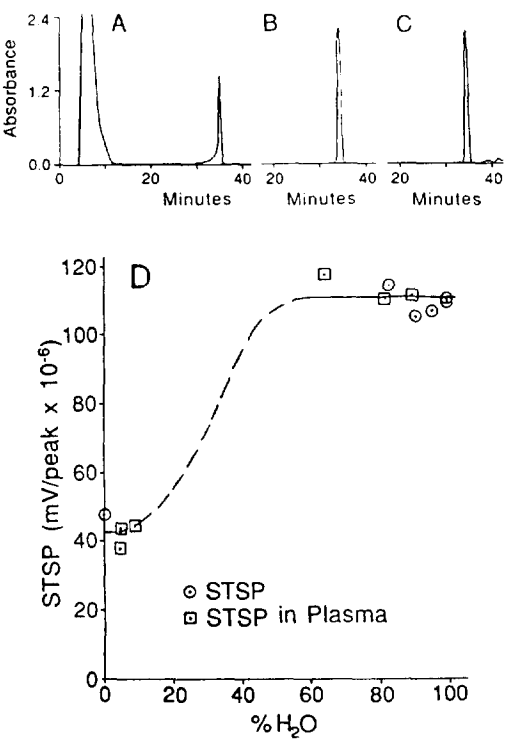


Fig. 3. Effect of solvent polarity on staurosporine loading on the reversed-phase HPLC column. A 25 μg Stsp/50 μl DMSO sample was added to (A) 1 ml acetone, (B) 11 ml aqueous 0.2% TFA, and (C) 1 ml acetone plus 10 ml aqueous 0.2% TFA. These samples were injected into the HPLC and the eluted components were detected by UV absorption at 292 nm. (D) Effect of the water composition of the sample on Stsp recovery from the reversed-phase $C_{18}$ column. The data at the lower end of the curve are for Stsp dissolved in >90% acetone. The data at the upper end of the curve are for Stsp dissolved in >64% aqueous 0.2% TFA. The precise shape of the curve between these data sets is unknown and is thus represented by the dashed line.

(Fig. 3C). This demonstrated that dilution of the acetone extracts of plasma would be necessary for quantitative loading of Stsp on the $C_{18}$ column.

The disadvantage of the 1:10 dilution was that it produced a large sample volume (11 ml). To determine if smaller dilutions could be used, the above experiment was repeated using 10:1, 5:1, and 2:1 dilutions. These experiments were conducted on samples where Stsp was added to plasma or to solutions of aqueous 0.2% TFA and acetone (Fig. 3D). It was found that the recovery

of Stsp from samples containing >90% acetone (<10% water) was only 39% of those diluted to >64% aqueous 0.2% TFA. The extraction of the Stsp from the plasma with the acetone appeared to be complete since the plasma acetone extracts gave the same quantitative results as the samples dissolved directly in acetone. From these results, we concluded that a dilution of one part acetone sample with two parts aqueous 0.2% TFA was sufficient to promote maximum adsorption of Stsp to the $C_{18}$ column. Therefore, the 2:1 dilution step was incorporated into the sample preparation protocol.

### *3.5. HPLC of Stsp in PEG, heparin, and EDTA*

At this point in the development of the HPLC method, the solvent used to dissolve Stsp was changed from DMSO to polyethylene glycol (PEG), which would cause less toxic effects in vivo. To determine if PEG interfered with Stsp chromatography, 3.5 $\mu$l PEG was dissolved in 1 ml aqueous 0.2% TFA and subjected to reversed-phase HPLC. During the acetonitrile elution, PEG could not be detected at 292 nm (Fig. 4A). Next, 10 $\mu$g Stsp in 10 $\mu$l PEG was dissolved in 100 $\mu$l plasma and the proteins were precipitated with 1 ml acetone. The proteins were removed from the acetone by centrifugation and the protein pellet was washed twice with 0.5 ml acetone which was added to the original acetone extract. This acetone sample was then diluted 2:1 with 0.2% aqueous TFA and the ca. 6 ml sample was injected into the HPLC. Stsp was eluted after 30 min and appeared in the quantity expected at 292 nm (Fig. 4B). From these experiments, it was concluded that PEG would not interfere with the analysis of Stsp.

Heparin and EDTA are commonly used to prevent clotting during blood collection and plasma preparation. To determine if heparin interfered with Stsp analysis, 100 $\mu$l 0.15 *M* NaCl containing 1000 units heparin/ml was dissolved in 1 ml aqueous 0.2% TFA. This 100-unit sample was mixed with 10 $\mu$g Stsp in PEG and subjected to HPLC. The Stsp peak detected at 292 nm was less than expected (Fig. 4C). While no heparin

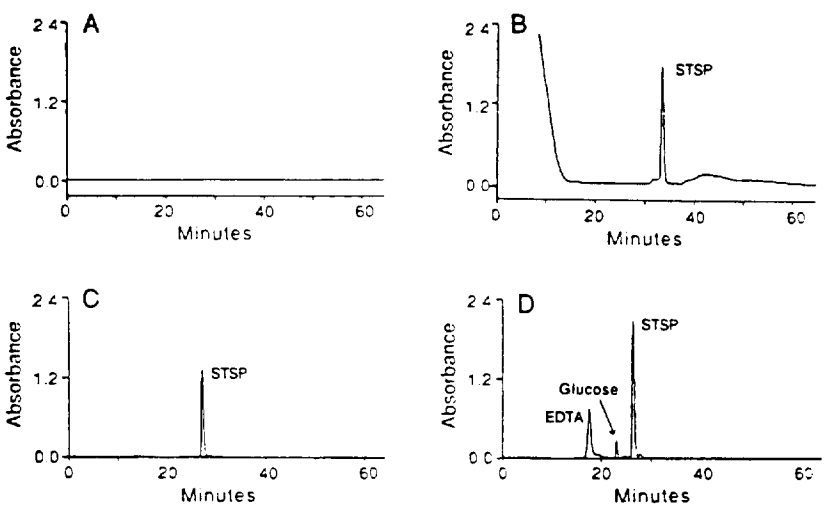


Fig. 4. Effects of PEG, heparin, and EDTA on the HPLC of staurosporine. All eluted components were detected at 292 nm. (A) A 1-ml sample of 3.5 $\mu$l PEG dissolved in aqueous 0.2% TFA was injected into the $C_{18}$ column. (B) A sample containing 10 $\mu$g Stsp/10 $\mu$l PEG was added to 100 $\mu$l plasma. The Stsp was recovered as an acetone extract which was diluted 2:1 with aqueous 0.2% TFA and injected into the $C_{18}$ column. (C) A 1-ml sample of aqueous 0.2% TFA containing both 100 units of heparin dissolved in 0.1 ml of 0.15 *M* NaCl and 10 $\mu$g Stsp dissolved in 10 $\mu$l PEG was injected into the $C_{18}$ column. (D) A 1-ml sample of aqueous 0.2% TFA containing both 0.3 mg EDTA dissolved in 0.1 ml 5% glucose and 10 $\mu$g Stsp dissolved in 10 $\mu$l PEG was injected into the $C_{18}$ column.

could be detected at this wavelength, a large heparin peak was detected at 215 nm eluting just ahead of Stsp (data not shown). Injection of four times less heparin (25 units) gave a similar size heparin peak detected at 215 nm, indicating that much of the heparin was not eluted from the column. Following these experiments, Stsp samples eluted from the column early (at 27 min), indicating the heparin had fouled the column irreversibly. From these experiments, it was concluded that the use of heparin should be avoided during blood sample collection.

Since EDTA can be substituted for heparin in the preparation of plasma, we investigated EDTA chromatography in the HPLC system. The 10.5 mg EDTA in a coated blood collection tube was dissolved in 3.5 ml of 5% glucose, and 100 $\mu$l of this solution (0.3 mg EDTA) was added to 1 ml of aqueous 0.2% TFA containing 10 $\mu$g Stsp dissolved in PEG. When this sample was subjected to HPLC, three peaks were detected at 292 nm eluting at 18, 23, and 26 min (Fig. 4D). These peaks were identified as EDTA, glucose, and Stsp respectively and the Stsp was recovered in the amount expected. It was concluded that the collection of blood and preparation of plasma could be accomplished using EDTA without interference of EDTA with Stsp analysis.

### 3.6. Quantification of Stsp in plasma samples

From the above experiments, we designed a protocol for Stsp analysis. The details of this protocol are described in the Methods section. To determine if the method gave quantitative results, plasma samples collected in EDTA were spiked with known quantities of Stsp dissolved in PEG. The plasma proteins were precipitated with acetone, the protein pellets were washed twice with acetone, and the combined acetone solutions were diluted 2:1 with aqueous 0.2% TFA. This 6-ml sample was pumped through the $\mu$Bondapak $C_{18}$ column followed by 5 ml of aqueous 0.2% TFA to rinse out the sample tube, pump, and pump lines. Following this loading procedure, the pumping of aqueous 0.2% TFA was continued to flush the acetone out of the column until the absorbancy of 292 nm was less than 1. The Stsp was then eluted with the linear acetonitrile gradient. For example, HPLC of a plasma sample containing 2 $\mu$g Stsp is illustrated in Fig. 5A. The Stsp peak area was determined by computer analysis (Fig. 5B). A calibration curve was constructed from such samples containing various amounts of Stsp (Fig. 5C). The calibration was linear with respect to $\mu$g Stsp

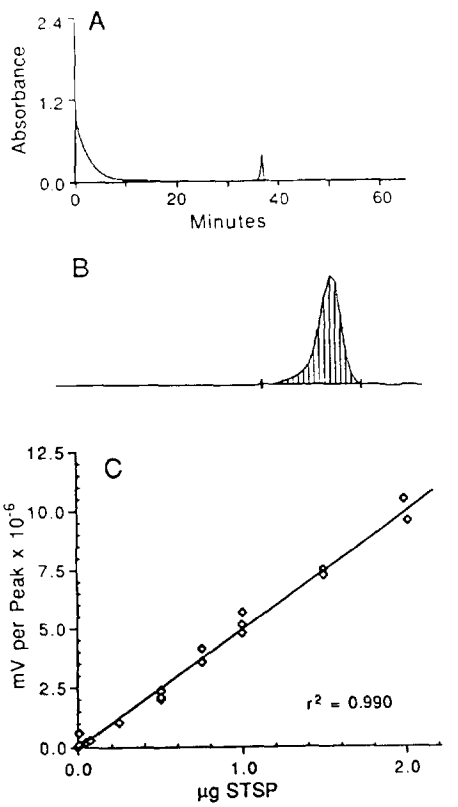


Fig. 5. Quantification of staurosporine by HPLC analysis. Plasma obtained from EDTA-treated blood was spiked with various concentrations of Stsp. The proteins of a 100-$\mu$l plasma sample were precipitated with acetone and the precipitate was washed twice with acetone. The pooled acetone containing the Stsp was diluted 2:1 with aqueous 0.2% TFA and subjected to HPLC. (A) HPLC elution of 2 $\mu$g Stsp detected by UV absorption at 292 nm. (B) Computer quantification of the Stsp peak eluted at 36.7 min. [$t_R$ = 36.70 min; area: 10 725 729; height: 347 461; start time: 35.60 min; end time 37.30 min.] (C) Calibration curve of the peak area (in accumulated mV/peak) vs. $\mu$g Stsp in the plasma sample. Least squares fit of replicate data had a correlation coefficient ($r^2$) of 0.990.

loaded on the column and the correlation coefficient ($r^2$) of this calibration curve was 0.990. The limit of detection was 0.1 $\mu$g Stsp.

To determine the precision of the method, triplicate plasma samples at four different Stsp concentrations were processed through the entire protocol and the results were subjected to statistical analysis as described by Natelson [26] for clinical samples. This analysis (Table 1) indicated that the standard deviation of the method is ±0.298. In the case of a 1 $\mu$g Stsp sample this standard deviation is ±5.8% of the mean. Thus, we can say that this method will yield results that are ±11.6% from the mean (2 standard deviations) 95% of the time. This is within the normal expected precision range for clinical samples of this nature [26].

The accuracy of the method for measuring only Stsp has been amply demonstrated. Processed plasma samples containing no Stsp produce no peak between 30 and 40 min on the HPLC chromatogram. The same peak is obtained for Stsp whether it is in aqueous solution (Fig. 2A) or in plasma (Fig. 2C). The same quantitative results are also obtained whether the Stsp is in aqueous solution or in plasma (Fig. 3D) demonstrating that the plasma components do not interfere with measurements of Stsp. Since we have shown the method to be specific, the precision of the method is also a statement of the accuracy of the method [26].

### 3.7. Stability of Stsp in frozen samples

It was anticipated that our experiments would require the storage of frozen plasma samples containing various amounts of Stsp before analysis could be performed. To determine if the Stsp was stable under such conditions, fresh plasma samples containing various amounts of Stsp were subjected to analysis and then frozen and stored at −25°C. At various times thereafter, the samples were thawed and reanalyzed (Table 2). These data indicated that Stsp was stable in frozen plasma and that no change in the Stsp analysis occurred outside the precision of the method over the course of 3 months in storage. This stability makes it possible to collect in vivo samples, prepare plasma immediately, and then freeze the samples for Stsp analysis at a later,

Table 1
Precision of measurements

| Stsp ($\mu$g) | $x$ mV·10 $^{6}$ | $x^2$ | $\Sigma x$ | $\sum x^2$ | $\frac{(\sum x)^2}{N}$ |
|---|---|---|---|---|---|
| 0.50 | 2.37 | 5.6169 | | | |
| 0.50 | 2.00 | 4.0000 | 6.70 | 15.0458 | 14.9633 |
| 0.50 | 2.33 | 5.4289 | | | |
| 0.75 | 3.58 | 12.8164 | | | |
| 0.75 | 4.11 | 16.8921 | 11.23 | 42.2401 | 42.0376 |
| 0.75 | 3.54 | 12.5316 | | | |
| 1.00 | 4.77 | 22.7529 | | | |
| 1.00 | 5.15 | 26.5225 | 15.57 | 81.1979 | 80.8083 |
| 1.00 | 5.65 | 31.9225 | | | |
| 1.50 | 7.48 | 55.9504 | | | |
| 1.50 | 7.28 | 52.9984 | 21.99 | 161.2217 | 161.1867 |
| 1.50 | 7.23 | 52.2729 | | | |

Samples/set = $N$ = 3, degrees of freedom/set = $N - 1 = 2$; $\Sigma(\Sigma x^2) = 299.7055$, $\Sigma(\Sigma x)^2/N = 298.9959$; $\Sigma(\Sigma x^2) - \Sigma(\Sigma x)^2/N = 0.7096$; total degrees of freedom = D.F. = 2/set · 4 sets = 8. Standard deviation = $\sigma = \sqrt{(\Sigma(\Sigma x^2) - \Sigma(\Sigma x)^2/N)/\text{D.F.}} = \sqrt{0.0887}$; $\sigma = \pm 0.2978$, $2\sigma = \pm 0.5956$, variance = $\sigma^2 = 0.0887$. At 1 $\mu$g Stsp, $\bar{x} = 5.19 \pm 0.60$ for $2\sigma$; i.e., this method will yield results that are ±11.6% from the mean 95% of the time.

Table 2
Effect of frozen plasma age on staurosporine analysis

| Sample No. | Fresh Stsp. (μg/sample) | Frozen Stsp (μg/sample) | Days frozen | Change after freezing (% of fresh sample) |
|---|---|---|---|---|
| 1 | 2.132 | 2.161 | 1 | 101.4 |
| 2 | 1.857 | 2.086 | 14 | 112.3 |
| 3 | 0.734 | 0.687 | 21 | 93.6 |
| 4 | 1.212 | 1.118 | 30 | 92.2 |
| 5 | 2.058 | 2.208 | 60 | 107.3 |
| 6 | 2.284 | 2.154 | 74 | 94.3 |
| 7 | 0.759 | 0.727 | 90 | 95.7 |
| 8 | 1.485 | 1.342 | 90 | 90.4 |
| 9 | 0.488 | 0.516 | 90 | 105.8 |
| 10 | 1.023 | 1.009 | 90 | 98.6 |

more convenient time following the in vivo part of an experiment.

### 3.8. Step in plasma of rats given Stsp in vivo

The Stsp assay developed above was used to analyze the level of Stsp in plasma after administering a single bolus injection of 0.2 mg Stsp dissolved in 0.2 ml of PEG into the jugular vein of a rat. If this amount of Stsp was uniformly distributed throughout the 10 ml blood volume of a rat and if the Stsp was distributed by passive diffusion throughout the volume of the RBC as well as the volume of the plasma, the concentration of Stsp would be expected to be 20 μg/ml in the plasma. Acetone extraction of a 0.1-ml aliquot of this plasma would result in a 2-μg Stsp load for HPLC analysis. A plasma sample spiked with 2 μg Stsp simulating this initial (time zero) condition is shown in Fig. 6A. If the Stsp did not penetrate the red blood cells and was thus confined to the 5-ml fluid plasma compartment, the concentration of Stsp in the plasma would be expected to be 40 μg/ml, resulting in an HPLC load of 4 μg Stsp (twice that shown in Fig. 6A). From these considerations, the analysis of Stsp in the in vivo samples of this experiment was expected to be within the operational range of the assay.

However, when blood samples were collected by cardiac puncture, 5 min or 15 min after injection of Stsp, no Stsp could be detected in the plasma (Fig. 6B and C, respectively). Thus, the levels of Stsp in the plasma were <0.1 μg per 0.1 ml sample, which is the detection limit of this assay (see calibration curve, Fig. 5C). This reduction of plasma Stsp from 40 μg/ml to <1 μg/ml in less than 5 min indicated that the Stsp was either being rapidly destroyed or transferred from the plasma compartment to some other compartment in vivo.

## 4. Discussion

In this work, we have developed a method for analysis of Stsp in blood plasma samples. The method has the advantage of using common chemical reagents and instruments available in most biochemistry laboratories. The method consists of two processes: the extraction of Stsp with acetone, followed by the purification of the Stsp by HPLC.

In the first process, the treatment of plasma with acetone accomplished two necessary operations in one step. It simultaneously precipitated the proteins out of the sample and extracted the Stsp into a solution free of interfering macromolecular contaminants. In the second process the reversed-phase HPLC also accomplished two necessary operations in one step. It concentrated the Stsp from the very dilute solutions and separated the Stsp from the low-molecular-mass reagents and blood components. The removal of

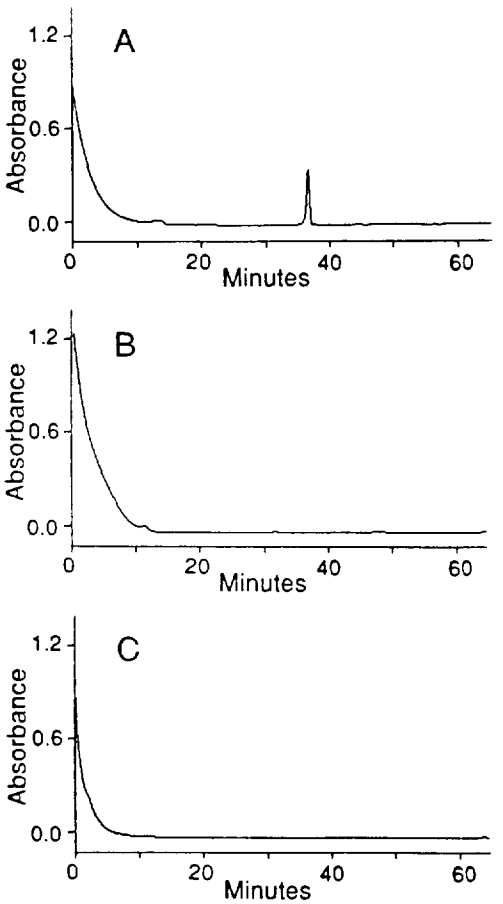


Fig. 6. HPLC analysis of staurosporine in the plasma of rats injected intravenously with staurosporine. (A) Analysis of plasma sample spiked in vitro with 2 $\mu$g Stsp. (B) Analysis of a 0.1-ml plasma sample withdrawn from a rat 5 min after administering a 200-$\mu$g bolus injection of Stsp. (C) Analysis of 0.1-ml plasma sample withdrawn from a rat 15 min after administering a 200-$\mu$g bolus injection of Stsp.

all these interfering substances facilitated the use of the absorbance peak of Stsp at 292 nm to detect and quantify the drug using a common variable wavelength flow spectrophotometer.

While the solubility of Stsp in acetone facilitated its extraction from plasma, this solubility was a hindrance during reversed-phase HPLC. The high solubility of Stsp in acetone reduced its interaction with the $C_{18}$ column, thus decreasing the recovery of the Stsp on the column. This problem was easily overcome by diluting the acetone extract 2:1 with water to increase the polarity of the sample solvent. Although this simple step increased the sample volume, the high efficiency of Stsp adsorption on the $C_{18}$ column under these more polar solvent conditions resulted in the total adsorption of Stsp on the column and in the concentration of Stsp into a sharp origin at the top of the column during the loading process. This resulted in a sharp elution peak which was concentrated enough to be detected by UV adsorption and easily resolved from reagents such as DMSO, EDTA PEG, and glucose used in the procedure.

Heparin, which is commonly used to prevent blood clotting, was found to elute near Stsp during HPLC. This crowding was not a problem for the analysis of Stsp, since heparin could not be detected at the 292 nm wavelength used to quantify Stsp. However, heparin was a problem because it fouled the HPLC column by irreversible adsorption. Thus, we have found that it is best to collect the blood samples in EDTA-treated tubes to prevent clotting. The EDTA elutes far ahead of Stsp and does not interfere with the analysis.

Since our work with Stsp is expected to involve a large number of samples in the future, it will be necessary to store plasma samples until they can be processed. For this reason, we compared the analysis of Stsp in fresh and frozen plasma. We found that samples could be frozen at −25°C for up to 3 months without any quantitative or qualitative changes in analysis.

The accuracy and precision of the method was found to be sufficient for the studies anticipated. The peak detected at 292 nm eluting from the HPLC column at 36 min was specific for Stsp. No blood or reagent components interfered with its quantification and the calibration curve demonstrated a linear response to Stsp concentration. Triplicate analyses demonstrated that the method will yield results that are ±11.6% from the mean 95% (2 standard deviations) of the time. This precision is within the range normally found for clinical samples [26] and will be sufficient for the experiments planned.

While this method does not provide an ul-

trasensitive analysis, we have found we can successfully use it to determine the fate of Stsp in blood. For example, when Stsp was injected into the bloodstream of rats, no Stsp could be detected in the plasma at 5 min after injection, even though enough Stsp was administered to be easily detectable if it was simply contained in the plasma. Thus, it must be concluded that in vivo some compartment other than the plasma must be adsorbing the Stsp from the plasma and sequestering it. Two likely candidates for this Stsp "sink" would be the red blood cells and the endothelium of the heart and lungs. These experiments suggested that the reason no G1 arrest was observed in the bone marrow of rats injected with Stsp [14] was that the Stsp never reached its target cells.

The next phase of this work is to determine what compartment of the body is absorbing the Stsp. We have used this method to determine that the red blood cells are a reversible carrier of the Stsp [27] and that the endothelium cells of the circulatory system are the likely Stsp "sink" [28]. Those studies are beyond the scope of this report and have been summarized in preliminary form elsewhere [27,28].

This method has also been used to determine the pharmacokinetics of Stsp in vivo. This became possible when we found that we could increase the sensitivity of detection by UV absorption at 292 nm. This was accomplished when the method described in this report was used to analyze Stsp in plasma following injections of Stsp in vivo. It was found that the Waters Model 490 UV flow monitor can detect Stsp eluting from the column even though the peak is too small to normally be recorded above background. These peaks were amplified by a post-chromatography computer program and quantified. The details of that work are also beyond the scope of this report and have been summarized in preliminary form elsewhere [28]. They are mentioned here to demonstrate that the acetone extraction and HPLC procedures reported here lay the foundation for further work on Stsp. Using this detection system in conjunction with the HPLC method developed in this report, we hope to determine the dose levels required to maintain an animal at 10 ng Stsp/ml plasma in order to induce G1 arrest in normal proliferating cells.

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