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# ANALYSIS OF THE ANTICANCER DRUGS VP 16-213 AND VM 26 AND THEIR METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

A rapid and convenient high-performance liquid chromatographic procedure for the analysis of the clinically useful anticancer agents VP 16-213 and VM 26 is described. The drugs, which are semi-synthetic derivatives of the natural product podophyllotoxin, are extracted from plasma with chloroform. The extracts are evaporated to dryness, reconstituted in methanol, and chromatographed on a reversed-phase microparticle $C_{18}$ column using isocratic elution with a mixture of methanol—water (60:40). Each drug is used as the internal standard for the other. Quantitation to 500 ng/ml (0.85 nmole/ml) plasma is based on peak height ratios using UV detection at 254 nm. Patient plasma concentration versus time data agree well with previously published data obtained using radiolabelled drug.

Investigations into the nature of the hydroxy acid metabolite of VP 16-213, carried out using paired-ion chromatography with tetrabutylammonium bromide and fluorescence detection, are described. Also, a unique separation of VP 16-213 and a possible metabolite, the isomer, picro VP 16-213, is described.

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## INTRODUCTION

VP 16-213 and VM 26 (Fig. 1), two semisynthetic derivatives [1] of the natural product podophyllotoxin, are currently undergoing promising clinical evaluation as anticancer agents [2]. The pharmacokinetic behavior of these drugs in patients has been extensively investigated by Creaven and Allen [3–5] and Pelsor et al. [6] using tritiated VP 16-213 and VM 26. In these studies, the tritiated drugs were extracted with chloroform from biological fluids and subjected to scintillation counting. A simple, rapid, routine and non-radioactive assay for these drugs in plasma was required for further clinical investigation of the pharmacokinetics and metabolism of these drugs. This has been accomplished using reversed-phase high-performance liquid chromatography (HPLC) on a microparticle $C_{18}$ column.

Pelsor et al. [6] and Allen et al. [7] have reported that VP 16-213 is extensively metabolized to a chloroform insoluble hydroxy acid (Fig. 2). They isolated this acid and characterized it by the mass spectrum of the permethylated derivative. This metabolite was further investigated by again utilizing reversed-phase HPLC. Since the *trans*-hydroxy acid of VP 16-213 has not been synthetized, studies proceeded with the easily synthetized *cis*-hydroxy acid. Since the hydroxy acid was poorly retained on the reversed-

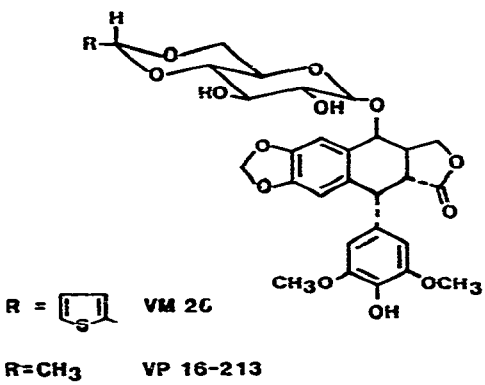



Fig. 1. Structures of VP 16-213 and VM 26.

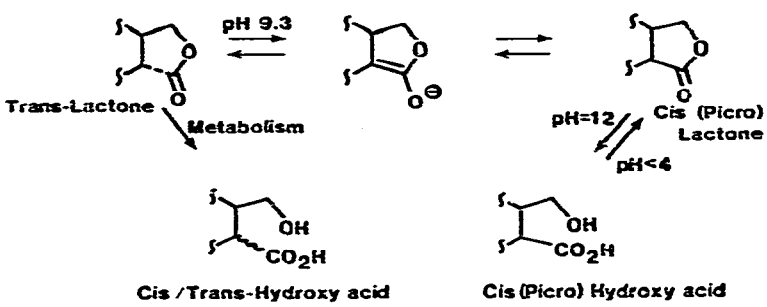



Fig. 2. Chemical interconversions of the lactone ring of the natural product podophyllotoxin and of derivatives such as VP 16-213 and VM 26 (all *trans*-lactones).

phase $C_{18}$ column, paired-ion chromatography was applied to the analysis. Fluorescence detection was employed to achieve specificity and sensitivity of analysis. Initial isolation of the presumed hydroxy-acid metabolite of VP 16-213 from plasma was by neutral adsorption on XAD-4 resin.

Reports have appeared in the literature [8] suggesting that podophyllotoxin undergoes epimerization in vivo to the much less biologically active picropodophyllotoxin (Fig. 2). This possibility for VP 16-213 was investigated. This necessitated the development of a separation of VP 16-213 from picro VP 16-213, Separation of VP 16-213 and its picro isomer could not be achieved on a reversed-phase $C_{18}$ column but was effected on a reversed-phase microparticle phenyl column.

## EXPERIMENTAL

### *Materials*

VP 16-213 and VM 26 were a generous gift from Drs. H. Friedli and H. Stähelin (Sandoz, Basle, Switzerland). Picro VP 16-213 and picro VP 16-213 hydroxy acid were synthesized as described below. Stock solutions (1 mg/ml) were made up in methanol and stored in a refrigerator at 5° for no longer than one week.

Methanol distilled in glass was obtained from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). Water was used as obtained from the building purified water system. Ethyl acetate and chloroform, CHROM-AR grade were purchased from Mallinckrodt (St. Louis, Mo., U.S.A.). Tetra-*n*-butylammonium bromide X grade was obtained through Scientific Products (McGaw Park, Ill., U.S.A.) from Eastman Chemicals (Rochester N.Y., U.S.A.). Ceric ammonium sulfate and 85% phosphoric acid were obtained from Fischer Scientific (Chicago, Ill., U.S.A.). XAD-4 resin was supplied by Supelco (Bellefonte, Pa., U.S.A.). Precoated thin-layer chromatography (TLC) plates of silica gel (0.25 mm) on glass with fluorescent indicator were obtained from EM Laboratories (Elmsford, N.Y., U.S.A.). Extractions were carried out in 16 × 125 mm culture tubes with PTFE-lined caps (Scientific Products). All glassware was routinely washed in chromic acid.

### *Apparatus*

A Waters Assoc. (Milford, Mass., U.S.A.) Model ALC 202 high-pressure liquid chromatograph equipped with a UV detector at 254 nm and a Model U6K injector was used. Fluorescence detection was accomplished by running the column effluent through a 35-$\mu$l flow cell (Aminco, Silver Springs, Md., U.S.A.) inserted in the cell holder of an Aminco-Bowman SPF spectrofluorometer. Analyses of VP 16-213 and its acid metabolite and of VM 26 were performed using a 30 cm × 3.9 mm I.D. column prepacked with $\mu$Bondapak $C_{18}$ (10 $\mu$m) from Waters Assoc. An identical column prepacked with $\mu$Bondapak Phenyl (10 $\mu$m, with a phenyl group chemically bonded to $\mu$Porasil at 16% by weight) from Waters Assoc. was used to analyze picro VP 16-213.

*HPLC conditions*

Analyses were performed with a flow-rate of 1.0—1.2 ml/min at an inlet pressure of 1600—2200 p.s.i. For the analysis of VP 16-213 and VM 26, the solvent was methanol—water (60:40) although this ratio was altered slightly (e.g., to 58:42) depending on $C_{18}$ column performance. The separation of picro VP 16-213 and VP 16-213 on the phenyl column was achieved using methanol—water (50:50). Paired-ion chromatography of the VP 16-213 hydroxy acids was accomplished by dissolving tetra-*n*-butylammonium bromide to the appropriate molarity directly in the mobile phase. All solvents were clarified with a 0.5-$\mu$m MF-Millipore filter (Millipore Corporation, Bedford, Mass., U.S.A.). Injection volume was generally 20 $\mu$l.

*Separation of VP 16-213, picro VP 16-213 and picro VP 16-213 hydroxy acid by thin-layer chromatography*

The compounds were spotted in methanol and the TLC plates were developed in ethyl acetate. The spots were dark under short-wave UV light and were also visualized with a 1% solution of ceric ammonium sulfate in 85% phosphoric acid (w/w). The reagent was diluted 50/50 volume for volume with water to facilitate spraying. The $R_F$ values were: VP 16-213, 0.57 (light red); picro VP 16-213, 0.49 (light orange—red); picro VP 16-213 hydroxy acid, origin to 0.03 (light red).

*Synthesis of picro VP 16-213*

Picro VP 16-213 was synthesized by using a modification of a known procedure for the conversion of podophyllotoxin to picropodophyllotoxin [9]. 16 mg of VP 16-213, 2.5 ml of 1.2 *M* sodium acetate and 3.75 ml of ethanol were heated together with stirring for 2.5 h at 75°. The reaction medium was about pH 9. The ethanol was removed with a nitrogen stream and the pH was adjusted to 6 with 1 *N* HCl. The product was extracted with 10 ml of chloroform and the extract volume was reduced to about 0.5 ml. The product was then precipitated with diethyl ether. The ether—chloroform layer was decanted and reduced in volume by slow evaporation to produce more product as fine needles (m.p. 210—212°). The product was pure by TLC and HPLC and its mass spectrum exhibited all of the major ions found in the mass spectrum of VP 16-213. Particularly diagnostic were *m/z* 588 ($M^+$) and *m/z* 382 ($C_{21}H_{18}O_7$; the aglycone fragment and base peak).

*Synthesis of picro VP 16-213 hydroxy acid*

To 10 mg of VP 16-213 and 1 ml of water were added exactly two equivalents of accurately standardized 0.1 *N* NaOH. The mixture, pH about 12, was heated with stirring at 45° for 50 min. After cooling to room temperature, two equivalents of accurately standardized 0.1 *N* HCl were added, causing a precipitate to form. The mixture was immediately lyophilized and the residue was repeatedly swirled with 2-ml portions of ethyl acetate until most of the product had been taken up. As the combined extracts were reduced in volume with a nitrogen stream, the product crystallized out (m.p. 182—186°, decomposition).

The product was pure by TLC and HPLC and was very polar as suggested by

its low $R_F$ and low retention on the reversed-phase column. Its retention time could be increased by using paired-ion chromatography at pH 7 with tetra-*n*-butylammonium bromide, indicating that it was acidic. The carbonyl stretching frequency had shifted from 1775 $cm^{-1}$ (lactone) to 1710 $cm^{-1}$. The mass spectrum showed no melcular ion but the base peak for the aglycone fragment appeared at *m/z* 400, indicating successful hydrolysis of the lactone ring. Permethylation [10] shifted the aglycone fragment base peak to *m/z* 442, which would be expected for the hydroxy acid. The permethylated product also revealed *m/z* 676 ($M^{\pm}$), *m/z* 645 ($M-CH_3O$) and *m/z* 644 ($M-CH_3OH$) Under acidic conditions the underivatized product recyclized to picro VP 16-213. These observations are consistent with the product being picro VP 16-213 hydroxy acid (*cis*-acid).

*Calibration and analysis of VP 16-213 from plasma*

To 1 ml of plasma in a culture tube were added 10 $\mu$l of VM 26 solution (1 mg/ml) as internal standard. The sample was extracted with 5 ml of chloroform by gentle rocking for 15 min. Following brief centrifugation approximately 4.5 ml of the chloroform layer were transferred to a conical centrifuge tube and evaporated to dryness at 40° with a nitrogen stream. Methanol (50 $\mu$l) was added and the tube was sealed and vortexed. The samples were centrifuged 5—10 min to precipitate any particulate matter and 20 $\mu$l of supernatant were injected onto the HPLC system with the $\mu$Bondapak $C_{18}$ column. A calibration curve from 0.5 to 30 $\mu$g/ml was established by analyzing spiked samples in triplicate and plotting peak height ratios of VP 16-213:VM 26, after subtraction of background values, versus plasma concentration of VP 16-213. Patient samples were analyzed similarly by splitting the collected plasma sample (usually about 2–3 ml) and carrying out duplicate analyses using the zero time sample as background.

*Analysis for picro VP 16-213 hydroxy acid*

Before use, XAD-4 resin (40—80 mesh) was extensively washed with 1 *N* NaOH, water, acetone, chloroform, methanol, 1 *N* HCl, water and methanol, respectively. XAD-4 columns were poured in methanol into disposable pipettes with methanol-washed glass-wool plugs. The resin was then rinsed with 10 ml of methanol and then 20 ml water. The columns were stored in water and were not allowed to run dry.

To 1 ml of plasma in a centrifuge tube were added 1.5 ml of methanol to precipitate proteins. The sample was vortexed and then centrifuged for 10 min at high speed in a clinical centrifuge. The supernatant was decanted into a test tube and the volume was reduced to less than 0.5 ml with a nitrogen stream at 40°. The sample was then diluted by addition of 1 ml of water and was loaded onto the XAD-4 column. After rinsing with 20 ml of water, the compound was eluted with 3 ml of methanol into a conical centrifuge tube. The sample was blown dry at 40° with nitrogen, reconstituted in 50 $\mu$l of methanol, sealed, vortexed, centrifuged and injected (20 $\mu$l) on the HLPC system.

*Analysis for picro VP 16-213*

Picro VP 16-213 was extracted from spiked plasma with chloroform, as with

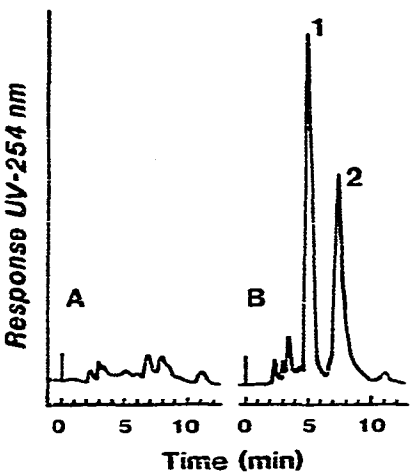
VP 16-213. Clinical samples were analyzed on a μBondapak Phenyl column to determine whether any discernable amount of picro VP 16-213 was present.

## RESULTS AND DISCUSSION

The separation and analysis of VP 16-213 and VM 26 are illustrated in Fig. 3B, a chromatogram of a patient plasma extract 2 h after infusion of VP 16-213. VM 26 was added as the internal standard. The two compounds are well resolved, appearing with retention times of 5 min and 7.5 min for VP 16-213 and VM 26, respectively. The plasma background at this sensitivity is low (Fig. 3A). Both compounds are essentially quantitatively extracted by this method.

The calibration curve from 30 μg/ml of plasma to the practical limit of detection of VP 16-213, 500 ng/ml (0.85 nmole/ml) by least squares analysis gave a linear correlation coefficient of $r^2 = 0.9998$. The calculated sample estimate of the coefficient of variation was on the order of 2.5% for each set of data values used to establish the standard curve.

The analysis of VP 16-213 in the plasma of a 30-year old man with testicular carcinoma is shown in Fig. 4. The patient received a constant i.v. infusion of 170 mg VP 16-213 over one-half hour. The patient was also receiving cyclophosphamide and cis platinum. The profile over 24 h agrees well with typical curves obtained by Allen and Creaven [4] and Pelsor et al. [6] using radiolabelled drug.



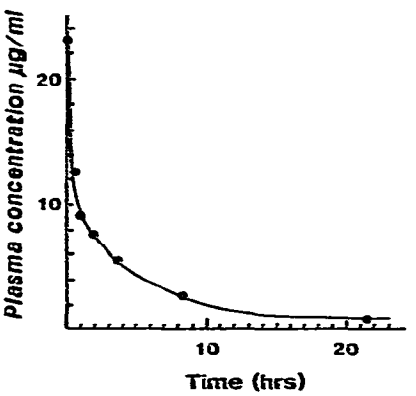




Fig. 3. HPLC UV traces of patient plasma samples treated by the chloroform extraction procedure. (A) Pre-infusion chromatogram; (B) 2-h post-infusion chromatogram with 10 μg/ml VM 26 as internal standard. Chromatographic conditions: 30 cm × 3.9 mm I.D. column with μBondapak $C_{18}$ (10 μm); solvent, methanol—water (60:40); flow-rate, 1.0 ml/min at an inlet pressure of ca. 1600 p.s.i. Peaks : 1 = VP 16-213 (9.5 μg/ml); 2 = VM 26 (10 μg/ml).

Fig. 4. Post-infusion plasma decay curve of VP 16-213 for a patient receiving the drug. Each point represents the average of two separate determinations from 1-ml plasma samples.

The described assay for VP 16-213 is directly applicable to VM 26. For the analysis of VM 26, VP 16-213 is used as the internal standard. The use of these drugs as complementary internal standards is possible since the drugs are not at present used in combination.

Some relevant transformations of VP 16-213, which are analogous to known reactions of podophyllotoxin [11], are summarized in Fig. 2. Reaction conditions to produce these analogs of VP 16-213 were modifications of similar reactions of podophyllotoxin. The *trans*-hydroxy acid cannot be produced by direct alkaline hydrolysis, since the compound rapidly epimerizes via the enolate to the *cis*-lactone and is then opened to the *cis*-hydroxy acid, i.e., the hydrolyzed picro compound. Picro VP 16-213 may be selectively produced at pH 9. However, although the *trans*-hydroxy acid of podophyllotoxin has been synthetized [12], no synthesis of the *trans*-hydroxy acid of VP 16-213 has been reported to date. Work therefore proceeded with the *cis*-hydroxy acid. At acidic pH values this compound rapidly recyclizes to picro VP 16-213. Therefore, pH-controlled extraction was not feasible. Thus, XAD-4 resin was used to adsorb the acid from plasma since neutral or mildly basic conditions were required. Recovery was greater than 90%. The resin required extensive cleaning, and the $C_{18}$ column was used to monitor the cleaning process. Even so, many UV absorbing interferences were adsorbed from plasma by the resin and subsequently eluted with methanol. Ion-pairing did not selectively move the acid away from the UV interferences. Therefore, fluorescence detection was examined.

Podophyllotoxins possess native fluorescence [13]. Picro VP 16-213 hydroxy acid can be excited at approximately 292 nm to fluoresce at 329 nm. By running the effluent from the HPLC system through a flow-cell inserted in the block of a typical spectrophotofluorometer, specificity for this acid was obtained. Because of the arrangement of the fluorescence detection system, the ultimate sensitivities available from fluorescence detection of this compound were not obtained, but low-microgram levels were easily measured. Paired-ion chromatography was also used at pH of about 6—7 to increase the retention time of the acid by adding tetrabutylammonium counter cation to the mobile phase. This helped to remove the acid from fluorescent interferences. A chromatogram of about 2 μg/ml of standard picro VP 16-213 *cis*-hydroxy acid is illustrated in Fig. 5B (broken trace), along with an extracted patient plasma sample obtained 2 h after infusion of VP 16-213. Background interferences (Fig. 5A) are low. Of interest is the observation that the picro VP 16-213 hydroxy acid standard has an ion-paired retention time of 5 min, whereas the metabolite has a retention time of only 4 min. Because the metabolite exhibited ion-pairing properties and its retention time does not match that of the *cis*-hydroxy acid (picro hydroxy acid), this constitutes indirect evidence that it may indeed be the *trans*-hydroxy acid. This implies that the hydrolysis may be enzymatically directed by, for example, an esterase. Assuming that the fluorescence response of the *cis*-hydroxy acid is comparable to that of the *trans*-hydroxy acid, and taking into account the 90% recovery from the XAD-4 extraction, the level of the hydroxy acid represented by the chromatogram of Fig. 5 is about 1—2 μg/ml of plasma. This agrees with the levels predicted by the pharmacokinetic model reported by Allen and Creaven [4] and Pelsor et al. [6]. This metabolism is currently being investigated further.

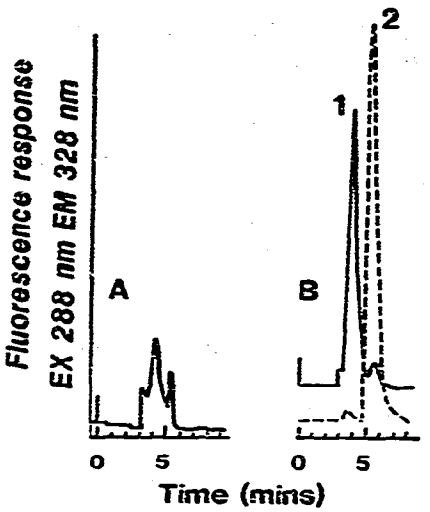



Fig. 5. HPLC fluorescence traces of patient plasma samples treated by XAD-4 extraction procedure. (A) Pre-infusion chromatogram; (B) 2 h post VP 16-213 infusion chromatogram. Chromatographic conditions: 30 cm × 3.9 mm I.D. column with μBondapak $C_{18}$ (10 μm); solvent, methanol—water (50:51) with 0.5 m*M* tetra-*n*-butylammonium bromide at 1.0 ml/min at an inlet pressure of ca. 2000 p.s.i. Peaks: 1 = metabolite of VP 16-213, presumed to be the *trans*-hydroxy acid compound; 2 = standard (2 μg/ml) picro VP 16-213 hydroxy acid (*cis*-hydroxy acid) run separately under the same conditions.

The fluorescence of these compounds can be applied not only to the analysis of the main metabolite but also to the analysis of the parent drugs. The chloroform extracts of plasma are fairly clean with respect to UV detection and extremely clean with respect to fluorescence detection. Therefore, it is not anticipated that background fluorescence will limit the ultimate sensitivity of detection greatly. Since with a UV detector 10 ng of VP 16-213 is easily seen on injection of pure compound, there is reason to expect that quantitation to at least this level with a dedicated flow-cell in a fluorometric detector designed for efficient light collection would be possible. This would then allow the analysis of these drugs in other biological fluids, such as cerebrospinal fluid, and tissue, where lower levels are encountered. It would also allow the analysis of the drugs in smaller collected volumes of plasma.

The separation of VP 16-213 and picro VP 16-213, obtained using a μBondapak Phenyl reversed-phase column, is shown in Fig. 6. The separation of these two components was not possible on a reversed-phase $C_{18}$ column. In view of the scattered reports [8] on the production of picropodophyllotoxin under biological conditions, the possibility of picro VP 16-213 being produced as a metabolite of VP 16-213 was investigated.

VP 16-213 and picro VP 16-213 are both very soluble in chloroform and have very similar chromatographic properties. Creaven and Allen [3] checked the purity of radiolabelled VP 16-213 extracted by chloroform from patient plasma. using several TLC systems. However, they did not use the TLC system which we have found to separate the two components (see Experimental). Further, they did not explicitly address the question of picro VP 16-213 as a potential

metabolite, at least in their publications. A slight possibility existed then that the pharmacokinetic data they derived were based on scintillation counting of VP 16-213 and picro VP 16-213 combined. Likewise, the assay on a $C_{18}$ column would have been unreliable because these two components are not separated in this system. However, to date, no picro VP 16-213 has been detected in clinical plasma samples.

In the event that VP 16-213 and VM 26 are ever used in combination therapy, as recently suggested [14, 15], the separation of picro VP 16-213 from VP 16-213 will be useful since the picro compound can be used as the internal standard and the assay can be conducted on a reversed-phase phenyl column.

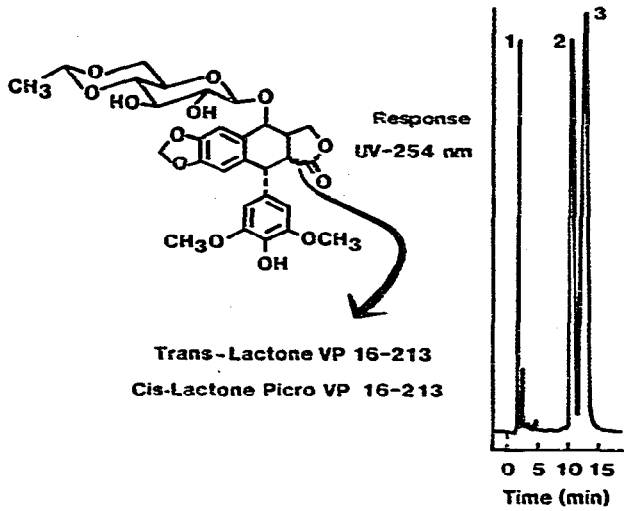



Fig. 6. HPLC separation of VP 16-213 (*trans*-lactone) and picro VP 16-213 (*cis*-lactone). Chromatographic conditions: 30 cm × 3.9 mm I.D. column with μBondapak Phenyl; solvent, methanol—water (50:50); flow-rate, 1.2 ml/min at an inlet pressure of ca. 2000 p.s.i. Peaks: 1 = picro VP 16-213 hydroxy acid; 2 = VP 16-213; 3 = picro VP 16-213.

## CONCLUSION

A rapid, practical assay is reported for the analysis of the promising anti-cancer agents VP 16-213 and VM 26. The assay is based on isocratic reversed-phase microparticle $C_{18}$ HPLC of chloroform extracts of plasma. Each drug functions as the internal standard for the other.

The acid metabolite of VP 16-213 appears to be the *trans*-hydroxy acid, although more definitive work is still needed.

Picro VP 16-213, a potential metabolite based on literature precedent, is apparently not produced in vivo.

Finally, the analytical potential of using detection of the native fluorescence of these podophyllotoxin derivatives is demonstrated.

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