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INVESTIGATIONS OF THE METABOLIC FATE OF TRITIATED VINCRISTINE IN THE RAT BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY*

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Abstract—A method is outlined for the separation of vincristine and its metabolites and/or degradation products using high-pressure liquid chromatography (HPLC). Using tritiated vincristine ([^{3}H]VCR) and this HPLC system, the metabolic fate of vincristine in the rat has been investigated. Although over 20 per cent of the injected dose (0.5 mg/kg; 25.8 μCi/kg) of radioactivity was excreted in the bile during the first 15 min, very little of this was in the form of metabolic products. At later time periods, less radioactivity was excreted in the bile, but a larger proportion of this radioactivity was in a form other than vincristine. In the urine, approximately 15 per cent of the radioactivity was in a form different from the injected solution. Although a large fraction of the radioactivity in the plasma did not chromatograph with vincristine, this may have been produced by decomposition, since a spiked control plasma had a similar pattern of distribution of radioactivity. Unfortified rat liver homogenates did not metabolize [^{3}H]VCR to any detectable extent. These results suggest that vincristine is not metabolized to a large extent in the rat.

Vincristine, a plant alkaloid of the vinca group, is a dimeric compound consisting of an indole and a dihydroindole nucleus. Investigations of the metabolic disposition of this drug have been hampered because reliable methods of isolating and quantitating vincristine and its metabolites were not available. In recent years, a method of preparing tritium-labeled vincristine has been developed [1–3]. Using [^{3}H]vincristine prepared by this method, preliminary data were obtained on the tissue distribution and excretion of vincristine in rats [3]. In this same study, attempts to determine the extent of metabolism of vincristine were inconclusive, since considerable degradation of the [^{3}H]vincristine occurred during the thin-layer chromatography procedure. The fate of vinblastine, which is structurally quite similar to vincristine, has been studied in greater detail in both animals [4–6] and in man [7]. Using thin-layer chromatography, Creasey *et al.* [8] concluded that vinblastine is excreted in the urine of the dog as the unchanged drug, while most of the drug in the stool was in the form of metabolites. With the emergence of high-pressure liquid chromatography (HPLC) as a powerful analytical tool, another obstacle to the investigation of the disposition of vincristine was removed. Previous attempts to use HPLC with vinblastine were not very successful [9]. However, with microparticulate columns, which have recently become available, separations can be performed much more efficiently. We report here an HPLC method for the separation of vincristine and its metabolites. Using [^{3}H]vincristine and this HPLC system, we have also investigated the metabolic fate of vincristine in the rat. The compounds excreted in the bile and urine have been separated and quantitated, as well as compounds present in plasma. Rat liver homogenates have been used in an attempt to show metabolism *in vitro*.

MATERIALS AND METHODS

Tritiated vincristine. The tritiated vincristine ([^{3}H]VCR) used in these studies was prepared by Dr. James P. Kutney of the University of British Columbia. The tritiation process employed catalytic exchange to selectively introduce the tritium into the indole aromatic rings of the vincristine molecule [1, 2]. This procedure produces labeled drug in which the tritium is in a stable configuration and not available for exchange. A similar tritiation process has recently been reported [3]. The tritiated vincristine was purified initially by recrystallization and finally by HPLC as described below. The vincristine was recovered from the purification processes in the form of the free base. The purified product had a specific activity of 42.5 mCi/m-mole. The radiochemical purity was 93 per cent as determined by HPLC. The [^{3}H]VCR was dissolved as the free base in ethanol and stored at 0°. Just prior to use, the [^{3}H]VCR solution was evaporated to dryness and redissolved in dilute sulfuric acid (1×10^{-4} N) to give a final concentration of 0.5 mg VCR/ml. This converted the vincristine base to vincristine sulfate.

Treatment of animals. Adult male Sprague–Dawley rats, 150–200 g, were allowed food and water *ad lib.* For urine collection, the animals were lightly anesthe-

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tized with ether, and [^{3}H]VCR (0.5 mg/kg; 25.8 μCi/kg) was injected i.v. into the tail vein. The rats were placed in separate plastic metabolism cages and urine was collected for 24 hr. As a control, urine was collected from untreated rats over the same time period. The urine from both groups was collected in separate vessels to which an aqueous solution containing 0.5 mg of non-radioactive vincristine base had been added. In addition, 50 μg [^{3}H]VCR was added to the control vessel prior to the start of the collection period. The purpose of the non-radioactive vincristine is to minimize decomposition of the tritiated compounds after they have been excreted in the urine (unpublished data). The purpose of the [^{3}H]VCR added to the control vessel was to estimate the amount of decomposition which occurred after the tritiated compounds had been excreted in the urine of the treated group. An aliquot of each urine was used to assay for total radioactivity. Both urines were adjusted to pH 8 with ammonium hydroxide and a 10-ml aliquot was extracted three times with 10 ml methylene chloride. The organic fractions were combined, evaporated to dryness under a stream of nitrogen at room temperature and immediately applied to the HPLC column as described below. An aliquot of the aqueous phase was assayed for total tritium.

For the determination of vincristine and its metabolites in the bile, rats were anesthetized with pentobarbital sodium (40 mg/kg) and the bile duct cannulated using size 10 polyethylene tubing. [^{3}H]VCR (0.5 mg/kg; 25.8 μCi/kg) was injected i.v. into the tail vein. Bile was collected over ice during several time intervals. Each collection vessel contained 0.25 mg of non-radioactive vincristine. To serve as controls, bile was collected from untreated rats into vessels containing 0.25 mg of non-radioactive vincristine and 50 μg [^{3}H]VCR. An aliquot of each sample was assayed for tritium. The bile samples were made basic to pH 8 with ammonium hydroxide and extracted three times with equal volumes of methylene chloride. The organic fractions were combined, evaporated and subjected to HPLC. The water-soluble fraction was assayed for total radioactivity and was not examined further.

For the determination of vincristine and its metabolites in plasma, rats were given an i.v. injection of [^{3}H]VCR (0.5 mg/kg; 25.8 μCi/kg). Ten min after the injection, the rats were anesthetized with ether and 5 ml blood was drawn by cardiac puncture into a syringe containing heparin and 0.25 mg of non-radioactive vincristine carrier. As a control, 5 μg [^{3}H]VCR was added to 5 ml of heparinized blood drawn from an untreated rat. This control blood was incubated at 37° for 10 min. At the end of the incubation period, 0.25 mg of vincristine carrier was added to the control blood. Both the control and experimental blood samples were centrifuged to obtain plasma. The plasma was drawn off, placed in a centrifuge tube and adjusted to pH 8 with ammonium hydroxide. The samples were then mixed with 5 ml of cold (0°) ethanol, centrifuged (5 min) and the supernatant was decanted. Each residue was extracted twice more with ethanol and the ethanol fractions were combined and extracted with an equal volume of methylene chloride. The organic phase was evaporated under nitrogen and applied to HPLC. The aqueous phase was counted for total radioactivity.

For the studies *in vitro*, rats were killed by cervical dislocation and the liver was quickly removed and placed on ice. Three g of each liver was homogenized in 12 ml of buffer solution, pH 7.4 (0.05 M Tris, 5.9 mM KCl and 114.9 mM NaCl). The incubation mixture consisted of 2.5 ml homogenate (0.5 g tissue) and 250 μg [^{3}H]VCR in 2.5 ml of the pH 7.4 buffer solution. Controls consisted of 2.5 ml of the same homogenate which was placed in boiling water for 5 min and cooled prior to the addition of 2.5 ml of buffer solution containing 250 μg [^{3}H]VCR. The mixtures were placed in a shaking incubator at 37° for 30 min. At the end of the incubation period the homogenates were poured into centrifuge tubes containing 5 ml of ice-cold ethanol and 0.25 mg of vincristine carrier. The solutions were adjusted to pH 8 with ammonium hydroxide and centrifuged. The supernatant was decanted and the tissue residue was extracted twice more with 5 ml of ice-cold ethanol. The ethanol fractions were combined and extracted three times with an equal volume of methylene chloride. The organic phase was evaporated under nitrogen at room temperature and chromatographed. The aqueous fraction was assayed for tritium. The tissue residue was combusted in a sample oxidizer and assayed for tritium.

Analytical methods. The bile and urine were assayed for total radioactivity by counting an aliquot of each in Aquasol scintillation mixture (New England Nuclear, Boston, MA). Total radioactivity in the homogenates and in the blood samples was determined by combusting an aliquot of each in a Packard model 306 samples oxidizer (Packard Instrument Co., Downers Grove, IL).

The separation of vincristine and its metabolites was performed on a Waters Associates (Milford, MA) model 202 liquid chromatograph. The column used throughout was a μBondapak C_{18} prepacked, reversed-phase column (Waters Assoc.). The solvent system consisted of a linear gradient going from solvent A (20% acetonitrile in 0.001 M K_2HPO_4 buffer, pH 7.5 to solvent B (80% acetonitrile in 0.001 M K_2HPO_4 buffer, pH 7.5). The program time was 40 min and the flow rate was 2.5 ml/min. A u.v. detector set at 254 nm was used to monitor the effluent from the column. The collection of fractions of column eluate was coordinated with observations of the u.v. monitor. Whenever a peak was indicated on the u.v. monitor, the entire elution volume of that peak was collected in a single fraction. These fractions were collected directly into scintillation vials. The eluted solvent was not evaporated, but was combined with 15 ml of scintillation mixture (Aquasol) and placed directly into the liquid scintillation spectrometer.

Scintillation counting techniques. Samples were counted in a refrigerated Packard model 2450 liquid scintillation spectrometer. The counting mixture used throughout was Aquasol (New England Nuclear, Boston, MA). Quench corrections were performed by means of an external standard.

RESULTS

The high-pressure liquid chromatogram shown in Fig. 1 illustrates the behavior of vincristine under the

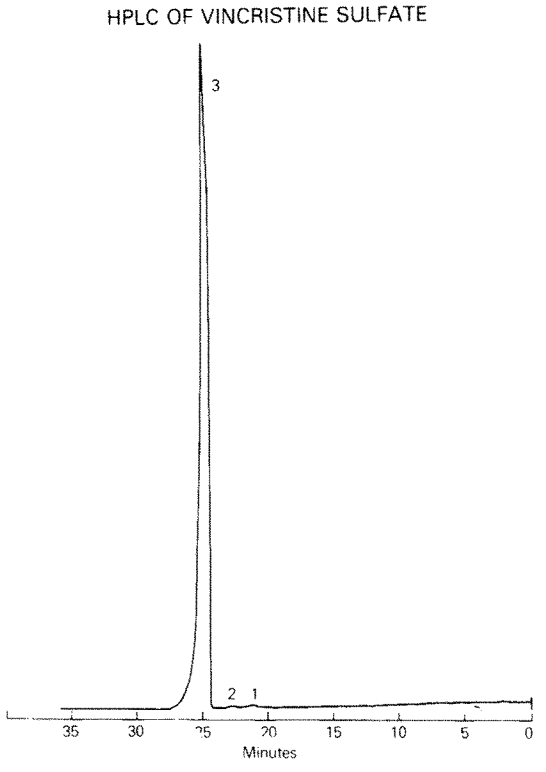


Fig. 1. HPLC of vincristine sulfate. Conditions: μBondapak C_{18} column; linear gradient 20–80% acetonitrile in water (0.001 M K_2HPO_4 pH 7.5, both solvents); time, 40 min; flow rate, 2.5 ml/min; chart speed, 0.2 in./min; u.v. detector set at 254 nm. Peak identities: 1 and 2 = impurities; peak 3 = vincristine.

conditions employed throughout these studies. The buffer used with the solvent systems (0.001 M K_2HPO_4) maintains a pH of approximately 7.5. The retention time of 25 min for vincristine is very reproducible and is independent of the initial form of the compound applied to the column (i.e. whether it is the free base or the salt). As seen in Fig. 1, there is no indication, such as a shoulder, that the major peak is composed of more than one compound. Two trace impurities (area 1 and 2) could be detected in the vincristine sulfate.

As shown in Fig. 2, the [^{3}H]VCR used in these studies was radiochemically approximately 93 per cent pure. Attempts to purify the compound further using HPLC met with only limited success. After several passes through the column, the maximum purity that could be obtained was 95 per cent. Two of the impurities in the [^{3}H]VCR (areas 1 and 2, Fig. 2) correspond to the impurities observed in the non-radioactive vincristine sulfate (areas 1 and 2, Fig. 1). A third impurity in the [^{3}H]VCR (area 4, Fig. 1) was not detectable in vincristine sulfate. The large area at the end of the chromatogram in Fig. 2 (area 5) represents an ethanol flush (5 ml) of the column to remove any residual radioactivity remaining on the column at the end of the run.

The HPLC system described above was used to determine the adequacy of the extraction procedure and the effect of storage conditions on the purity of the [^{3}H]VCR. When [^{3}H]VCR was added to water and the pH adjusted to approximately 8.0, greater than 99 per cent of the radioactivity was extracted into the organic phase by the procedure used in these studies (i.e. three separate extractions with methylene chloride). Analysis of this organic phase by HPLC indicated that no detectable decomposition had occurred. The overall recovery was greater than 98 per cent of the starting material. Thus, the procedures outlined here are shown to be adequate for the investigation of the metabolic fate of vincristine. The purified [^{3}H]VCR was dissolved in 95 per cent ethanol and stored at 0°. Under these conditions, decomposition occurs at the rate of 1–2 per cent/month. If stored in dry form, the decomposition rate is 5–10 per cent/month.

In the studies to be described below, separate chromatograms were performed for the control group and for the experimental groups. For each experiment, only one chromatogram is presented here since in most cases the chromatograms for the control group were identical to the experimental group. It should also be pointed out that, since we chose to collect fractions based on discrete peak areas rather than on elution volumes, the numbered areas of one chromatogram do not necessarily correspond to the same numbered areas from other chromatograms.

Urine collected over a 24-hr period after an i.v. injection of [^{3}H]VCR contained 12 per cent of the injected radioactivity. Approximately 10 per cent (i.e. 1.2 per cent injected label) of the radioactivity in the urine remained in the aqueous phase after extraction with methylene chloride. However, the aqueous phase from the control urine also contained about 10 per cent of the total radioactivity, suggesting that these are not water-soluble metabolites of vincristine, but result from imcomplete extraction of the urine by the methylene chloride. As shown in Fig. 3, there are a number of u.v.-absorbing compounds excreted in the urine. However, several of these areas on the chromatogram contain very little radioactivity (areas 1, 2, 3, 8, 9, 10 and 11), suggesting that they are not derived from vincristine but are natural components of the urine. Area 11 of the chromatogram represents the compounds which were eluted by the ethanol flush of the column. The vincristine area (area 6, Fig. 3) is very large in comparison to other areas since the carrier vincristine added before the extraction procedure appears in this area. The only major difference in the distribution of radioactivity on the chromatogram occurs in area 5 (Fig. 3), where the experimental group was more than three times the control group.

Since biliary excretion is the major route of elimination of vincristine in the rat [3, 11], the amount of vincristine and its metabolites in the bile was studied at several different time periods. The aqueous fraction from the extraction procedure was not qualitatively different from the control group at any of the time periods. Although over 20 per cent of the injected dose was excreted in the bile during the first 15 min, very little of this amount was in the form of metabolites. Almost 89 per cent of the radioactivity extracted from the 15-min bile was in the form of vincristine, compared to 92 per cent for control. There were, however, two other areas of the chromatogram (area 6 and 10) in which the experimental group differed from the control group. Between 15 and 60 min about 20

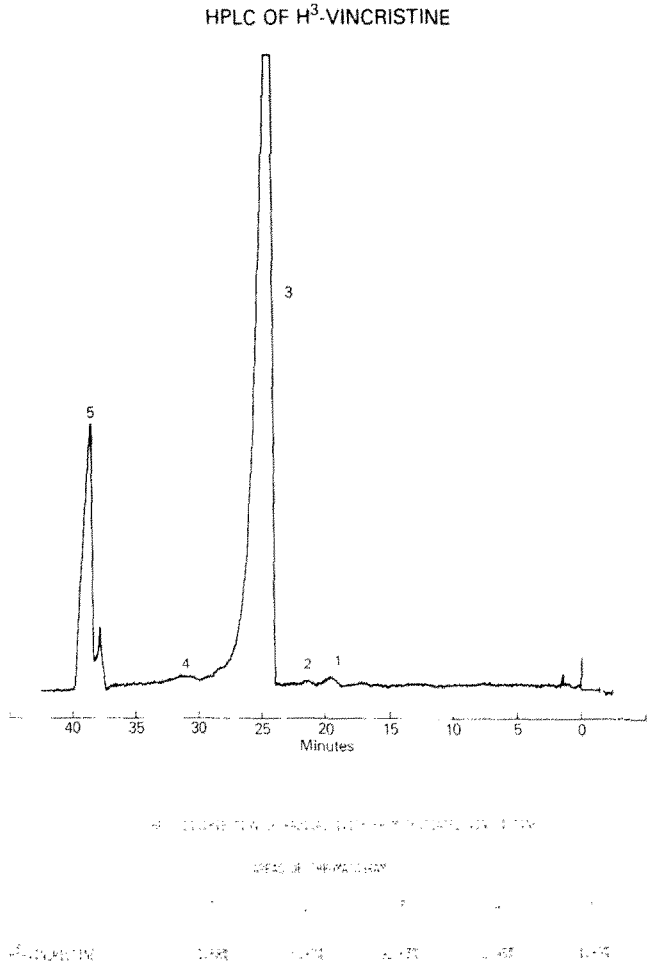


Fig. 2. HPLC of [^{3}H]vincristine. Conditions are the same as in Fig. 1. Peak identities: peaks 1, 2 and 4 = impurities; peak 3 = vincristine; peak 5 = ethanol rinse of column. Percentages refer to the per cent of total radioactivity recovered from the chromatogram.

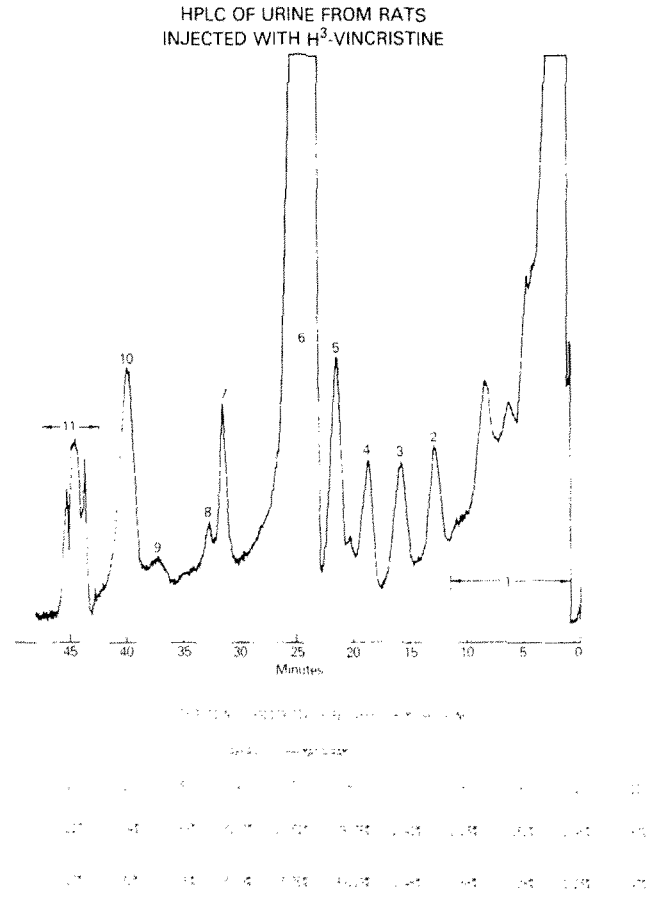
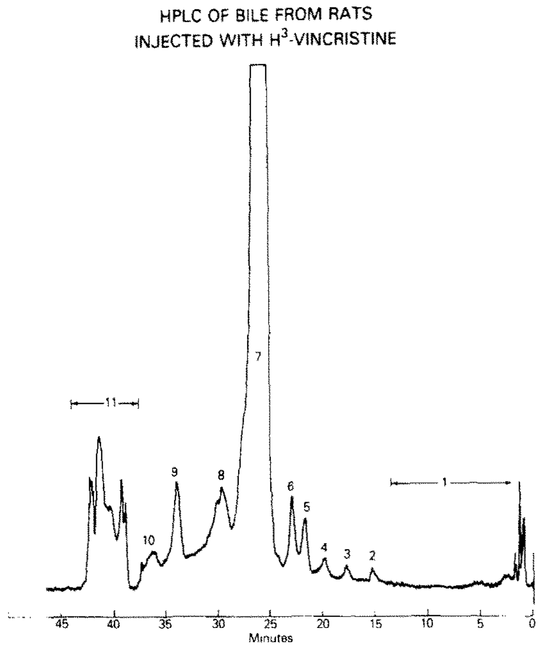


Fig. 3. HPLC of urine extracts. Conditions are the same as shown in Fig. 1. Peak 6 is vincristine; others are unidentified. Percentages refer to the per cent of total radioactivity recovered from the urine.



DISTRIBUTION OF RADIOACTIVITY RECOVERED FROM THE BILE

	AREAS OF CHROMATOGRAM										
	1	2	3	4	5	6	7	8	9	10	11
0-15 MIN	0.29%	0.10%	0.33%	0.32%	[illegible]	4.09%	88.68%	0.46%	0.20%	3.06%	[illegible]
15-30 MIN	0.64%	0.08%	0.40%	3.22%	3.75%	4.56%	82.37%	3.00%	1.00%	3.67%	[illegible]
30-60 MIN	[illegible]	0.20%	0.62%	0.20%	3.91%	4.88%	81.73%	1.45%	0.80%	[illegible]	[illegible]
CONTROL	0.12%	0.05%	0.20%	0.16%	2.23%	2.42%	91.93%	1.89%	0.75%	[illegible]	[illegible]
60-120 MIN	0.44%	0.15%	0.40%	0.19%	2.96%	4.71%	75.15%	2.44%	[illegible]	3.80%	4.78%
120-240 MIN	0.34%	0.12%	2.46%	0.26%	2.45%	2.94%	73.70%	2.25%	1.61%	[illegible]	[illegible]
CONTROL	0.42%	0.07%	0.22%	0.07%	[illegible]	8.26%	74.98%	1.92%	0.45%	2.72%	[illegible]

Fig. 4. HPLC of bile extracts. Conditions are the same as shown in Fig. 1. Peak 7 is vincristine; other peaks are unidentified. Percentages refer to the per cent of total radioactivity recovered from the bile.

per cent of the injected radioactivity was excreted in the bile. During this time, the radioactivity appearing in the vincristine peak (area 7, Fig. 4) was approximately 10 per cent less in the experimental group than in the control group. Between 1 hr and 4 hr another 10 per cent of the injected dose was excreted in the bile. Although the amount of this radioactivity which appeared in the vincristine peak (area 7, Fig. 4) was not different from the control group, there were differences in other areas of the chromatogram (e.g. areas 10 and 11 were greater than control).

When [^{3}H]VCR was incubated with whole blood for 10 min and the plasma extracted, only 67 per cent of the radioactivity could be recovered as vincristine, compared to 57 per cent in the experimental group. Two major areas of difference between the two groups were areas 3 and 8 (Fig. 5).

As seen in Fig. 6, there did not appear to be any differences in the distribution of radioactivity on the chromatogram from fresh liver homogenates compared to boiled controls.

DISCUSSION

Although vincristine has been used clinically for many years, very little is known about its pharmacokinetic behavior. The usual therapeutic dose of vincristine is relatively small (0.01 to 0.05 mg/kg) so that detection and quantitation of the drug and its metabolites in biological fluids have not been possible. The problems of producing a radiolabeled form of vincristine have been overcome in recent years, resulting in a synthetic process which is suitable for the production of relatively large amounts of tritiated vincristine [1–3]. Tritiated vincristine prepared by this method has been used to obtain some preliminary data on the distribution and excretion of vincristine in rats [3]. The tissue distribution and excretion of [^{3}H]vincristine in rats and dogs have been reported recently in greater detail in imvestigations performed in this laboratory [10]. These latter studies suggested that the radioactivity in the urine and bile of rats was almost entirely in the form of unchanged vincristine,

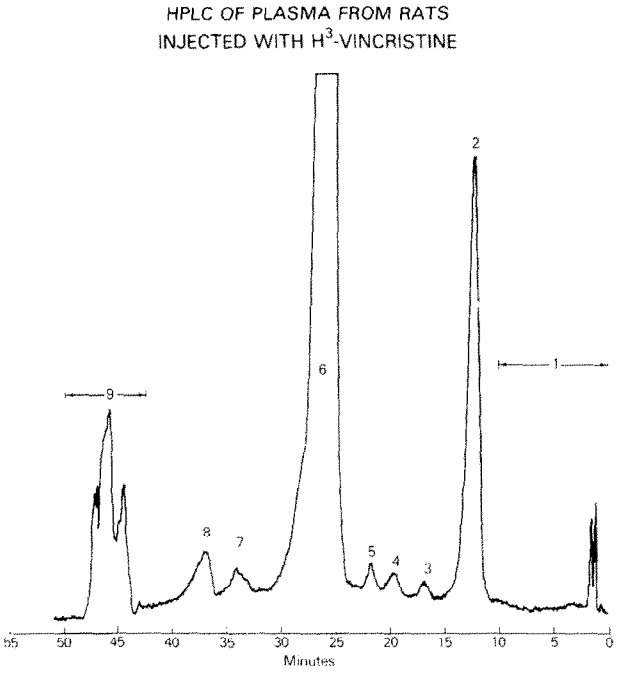


Fig. 5. HPLC of plasma extracts. Conditions are the same as shown in Fig. 1. Peak 6 is vincristine; other peaks are unidentified. Percentages refer to the per cent of total radioactivity recovered from the plasma.

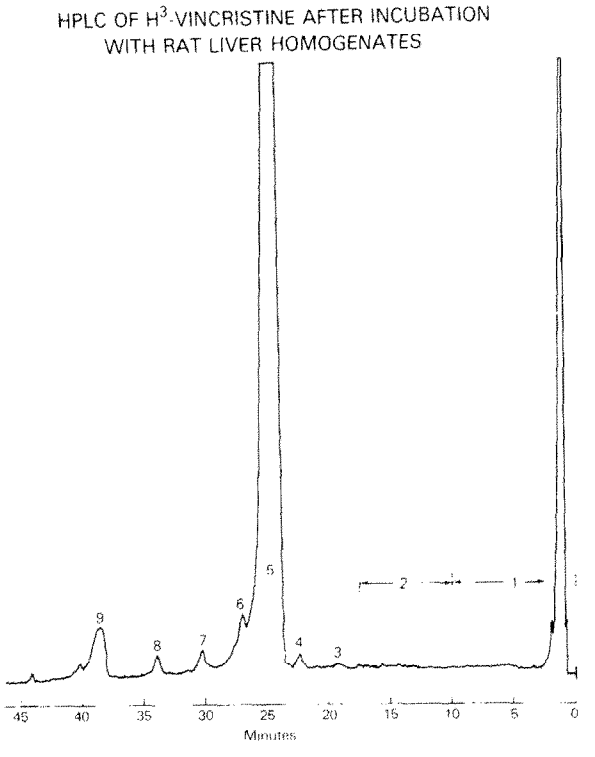


Fig. 6. HPLC of extracts of rat liver homogenates. Conditions are the same as shown in Fig. 1. Peak 5 is vincristine; other peaks are unidentified. Percentages refer to the per cent of total radioactivity recovered from homogenates.

while a considerable amount of the radioactivity in the plasma appeared to be metabolites of vincristine. However, the thin-layer chromatography system used in these studies has a number of disadvantages when used with vincristine. Since vincristine in the form of the free base can undergo decomposition, it is desirable to have a method in which the analysis is as rapid as possible. In addition, resolution of the various products of vincristine metabolism and/or decomposition is not possible with thin-layer chromatography.

In the present investigations, we describe a method for the separation of vincristine and its metabolites using high-pressure liquid chromatography. This technique has several advantages over the thin-layer chromatography systems utilized in the past. Since HPLC is a nondestructive process, the compounds applied to the column can be recovered in their original form. This allows HPLC to be used for the purification of mg quantities of [^{3}H]vincristine. The HPLC procedure can be carried out much more rapidly than thin-layer chromatography, thus minimizing decomposition of the vincristine. Furthermore, when the HPLC system is used in conjunction with a scanning u.v. detector, a complete u.v. spectrum can be obtained for each compound. Since the u.v. spectrum of vincristine and of related bisindole alkaloids is a summation spectrum of the two monomeric units in the molecule, any cleavage of vincristine to its monomers would result in entirely different spectra characteristic of either indole or dihydroindole units (see for example Ref. 11 and references cited therein). In addition, the HPLC characteristics of the monomers would be distinct from vincristine. We did not observe a spectrum in the products derived from vincristine other than that characteristic of a bisindole configuration and we, therefore, conclude that no significant breakdown to the monomer level had occurred.

The radioactivity excreted in the urine over a 24-hr period was found to be primarily in the form of unchanged vincristine (Fig. 3). The major difference between the treated group and the control group occurred in the area of the chromatogram just before the elution of vincristine (area 5, Fig. 3). The u.v. spectrum of this compound was almost identical to that of vincristine, suggesting that the dimeric nature of the molecule remains intact. According to the rules of reverse-phase chromatography, the elution time increases as the polarity of the compounds decrease. As seen in Fig. 3, very little radioactivity could be detected in the eary portions of the chromatogram from the urine. This suggests that very little of the [^{3}H]vincristine has been converted to polar metabolites. The compound which eluted just before vincristine (area 5, Fig. 3) would appear to be only slightly more polar than vincristine.

The extent to which vincristine is metabolized in the rat if difficult to determine from the data obtained from biliary excretion of the drug. Although the amount of radioactivity appearing in the vincristine peak was less in the experimental group than in the control, this does not necessarily indicate that metabolism has occurred. This decrease in radioactivity in both groups could be due to decomposition rather than metabolism. Even if decomposition is occurring, we cannot be certain that it is proceeding at the same rate in both groups. Furthermore, the decomposition products formed in the experimental group could themselves undergo metabolism to form different products than are seen in the control group. For example, the relatively large amount of radioactivity in area 10 of Fig. 4 could result from metabolism of one or more decomposition products rather than from metabolism of vincristine itself. When the radioactivity appearing in the bile was applied to HPLC, the results again suggested that vincristine was not metabolized to any great extent. During the first 4 hr after i.v. injection of [^{3}H]vincristine, over half of the injected radioactivity was excreted in the bile. Over 83 per cent of this radioactivity was in the form of unchanged vincristine. Since the initial purity of the [^{3}H]vincristine was approximately 93 per cent the extent of conversion to metabolites was not very great even if one assumes that no decomposition has taken place during this time. The results from the control group suggest that some decomposition probably has occurred in the experimental group. Thus, the extent of metabolism of the [^{3}H]vincristine is most likely very small (i.e. less than 10 per cent).

The studies with rat liver homogenates offer further evidence that vincristine is not metabolized to any great extent. After incubation for 30 min, no differences could be detected between boiled control homogenates and fresh homogenates.

Studies designed to isolate and quantitate the amounts of vincristine and its metabolites in the plasma were inconclusive. Vincristine is very rapidly cleared from the blood [3, 10]. Therefore, it was necessary to obtain the blood at an early time period (10 min after injection) in order to have enough radioactivity for application to HPLC. As reported previously with thin-layer chromatography [10], considerable amounts of radioactivity were recovered from areas other than the vincristine area. However, in the control group, in which [^{3}H]vincristine was incubated with whole blood for 10 min, only 67 per cent of the radioactivity could be recovered as vincristine. This would seen to indicate that a considerable amount of decomposition of [^{3}H]vincristine has occurred during the analytical procedure.

In conclusion, the data derived from the present studies are in agreement with previous observations that vincristine is not metabolized to a large extent by the rat [3]. It appears likely that the decomposition found with the control samples is also occurring *in vivo*. In this case, a significant amount of these products seems to be present in the rat. These compounds, even though not derived from an enzymatic process, could still be important in the mediation of the therapeutic and/or toxic effects of vincristine. It would be interesting to compare the biological effects of some of these compounds with vincristine.

The lack of metabolism of vincristine observed in the rat may be of considerable significance if confirmed in humans. These investigations are in progress in our laboratory.

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