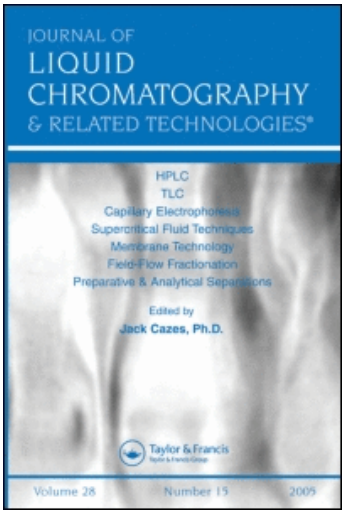


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# Analysis of Antitumor Antibiotics by High Pressure Liquid Chromatography (HPLC)

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# ANALYSIS OF ANTITUMOR ANTIBIOTICS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

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Cancer chemotherapy is a concern of many people today: laboratory scientists, clinicians, cancer victims and news reporters. Their hopes for a cancer cure are always raised when a new chemotherapeutic agent is discovered in the laboratory and shows some promising characteristics towards clinical application. Some of the agents that have been discovered are of natural origin[1], and are complex structures of unknown composition; others are enzymes or antibiotics. The antibiotics are obtained mostly from fermentations, but are also derived from plants or marine animals. Considerable efforts are being made in laboratories to purify these antibiotics to homogeneity and to determine their structure, mode of action, toxicity and applicability to clinical cancer chemotherapy. All these complex functions require analytical support. One of the most modern analytical tools, high pressure liquid chromatography



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(HPLC), is used with increasing frequency to analyze these antitumor antibiotics in different media and for different reasons.

Before the advent of HPLC, analytical methods used in connection with different antibiotic assays relied on liquid chromatography, countercurrent distribution[2], thin layer chromatography[3] and gas chromatography[4]. Liquid chromatography, in general, is a time-consuming, slow technique. Thin layer chromatography is much faster and uses inexpensive instrumentation but lacks resolution and reproducibility for complicated, large compounds and especially for mixtures. Gas chromatography is a fast chromatographic system, with good reproducibility, precision and specificity at relatively modest instrumentation costs. However, many antibiotics cannot be analyzed by gas chromatographic techniques because they cannot be volatilized.



In the last decade or so, HPLC has been developed into an analytical tool that combines the advantages of all previous chromatographic techniques. Most of the details of the techniques of HPLC are well described in recent literature[5][6], and there is no need to deal with them here. Briefly, HPLC uses a narrow column with small diameter column packing particles, high pressure to obtain the flow rate necessary for short analytical time and highly sensitive detectors. The short analytical time is an important factor in

analyzing antibiotics, because some antibiotics decompose during lengthy analytical manipulations. Also, analytical results may be needed urgently, e.g., for assessment of antibiotic composition in fermentation broth or in human or animal tissues. This short analytical time is achieved by high volume of solvent flow, which in turn is achieved by high column-inlet pressure. This analytical arrangement is very seldom a problem in connection with antibiotic analysis.

The separation of antibiotics is monitored with ultraviolet absorption detectors in most cases, since many of the antibiotics absorb in the ultraviolet range. However, some antibiotics, e.g., the aminoglycosides, have no characteristic ultraviolet absorption above 210 nm. In such cases pre- or postcolumn derivatization is performed to provide antibiotic derivatives which can be monitored by fluorescence or ultraviolet detectors.

HPLC has been used increasingly in the antibiotic field in research, quality control and manufacturing environments. For many antibiotics the official or most accepted assay is now performed by HPLC. However, some of the HPLC assays lack precise characterization of the chromatographic system, mostly because the system was applied to non-routine testing. For example, no precise control of the temperature is mentioned in most literature although work done with the vinca alkaloids demonstrated the importance of this parameter[7]. Precise

characterization of the columns used in connection with antibiotic work is almost entirely lacking. Today HPLC is used in the isolation studies of new antibiotics, in preparation of large quantities of antitumor antibiotics for biological studies and, if the assay proves to be sensitive enough, for quantitative estimation of antibiotics in biological fluids and in various drug preparations.

Because some of the work was done with equipment not used any more, these systems need to be translated to more current conditions. Routine assays should undergo collaborative studies before wide-scale introduction, and such studies were done for several antibiotic assays as discussed below.



This review summarizes and comments on the HPLC systems used in connection with antitumor antibiotic analysis. It is intended to alert the reader about possible use of HPLC in connection with studying antibiotics rather than as a critical evaluation of work done in other laboratories. Discussions are grouped according to the most important or most frequently analyzed antibiotics and the use of HPLC in isolation studies of new antitumor antibiotics. Moreover, the literature on determination of these compounds in biological fluids has been selected so that similar analytical details are not repeated too frequently. Also, methods older than 8 to 10 years are not reported in this discussion. It will be apparent to the reader that most of the HPLC processes discussed below use reversed

phase systems. The most frequently used column is μBondapak $C_{18}$. Some other columns used are Lichrosorb, Porasil, Ultrasphere ODS, Nucleosil $C_{18}$ and Durapak. Occasionally the application of a guard column like CO:Pell ODC or RSiL had to be employed. For eluting solvents combinations of methanol and acetonitrile with phosphate buffers are used most frequently.

It should be mentioned that in this review the HPLC nomenclature used is that of the original authors. It was felt that an attempt to modify their descriptions for the purpose of uniformity would create a problem for those who want to refer to the original paper. Also, no attempt was made to describe the origin and quality of reagents used in the experiments. It was assumed that all solvents were filtered and degassed before use. Structures of the discussed antibiotics can be found in the Merck Index, Handbook of Antibiotic Compounds (J. Berdy, A. Aszalos, M. Bostian and K. L. McNitt eds., CRC Press) and in the original articles cited, and have not been duplicated in this paper. Many papers describe the use of HPLC in conjunction with the objective of that paper, e.g., isolation of an antibiotic, metabolic studies, etc. In each case only the HPLC portion of the paper and the objective of the study have been described; details and results of the study were not reviewed here.



## ACTINOMYCINS

One of the earliest antibiotic groups discovered was the actinomycins. This family of antibiotics comprises closely

related chromopeptides which differ only in their amino acid composition[8]. Different chromatographic systems applied to separate these closely related antineoplastic antibiotics helped in the elucidation of their structure[9] and in their biological evaluation[10]. It was realized in the early separation attempts that distribution chromatography is an excellent method for separation of these partially lipophilic antibiotics[11] [12]. As a natural consequence, separation of the actinomycin mixtures was attempted by reversed phase HPLC.



In a successful HPLC separation of the actinomycins, Rzeszotarski and Mauger[13] used the following conditions: chromatograph, Waters Associates AL C202/6000 psi; UV detector, operated at 254 nm; column, 6 ft. x 1/8 in., filled with μBondapak $C_{18}$/Corasil or Bondapak phenyl/Corasil, stationary phases covalently bound to Corasil, columns drypacked by vibration and topping; mobile phase, water-acetonitrile (1:1); flow rate, 1 ml/min (1000 psi).

The above experimental conditions provided almost complete baseline separation for the actinomycin mixture $C_1$, $C_2$ and $C_3$ with symmetrical peaks and for the actinomycin mixture whose members contained cis-4-chloro-L-proline. This latter complex was obtained by using cis-4-chloro-L-proline as precursor in the fermentation medium of the microorganism of Streptomyces parvullus. This HPLC method provided evidence for the formation of the two new types of actinomycins, $CP_3$ and

$CP_2$. By using chromatographic recycling techniques, the major components of the latter complex could be prepared in sufficient quantities for chemical characterization.

A recent method used in several laboratories to determine quantitatively the actinomycin D content of bulk preparations (unpublished) utilizes the following conditions: chromatograph, Waters Associates Model 244; UV detector, operated at 254 nm; column, 30 cm x 4 mm, packed with μBondapak $C_{18}$; mobile phase, acetonitrile-water (6:4); flow rate, 2.5 ml/min; samples, about 0.25 mg/ml standard or bulk preparation of actinomycin D; calculations, actinomycin D content of bulk preparations calculated on the basis of comparison of the area under the peak of standard and samples. The above procedure was the subject of collaborative studies (unpublished). Statistical evaluation of these studies indicated the acceptance of this HPLC system for official analytical purposes.

The two HPLC systems described above make it possible to detect and separate common actinomycins and to quantitate actinomycin D in bulk preparations. For other determinations, such as quantitations of actinomycin in different tissues, no accepted method has yet been reported in the literature. Such assays are currently done by different techniques, like fluorescence spectroscopy. However, with the present sensitive detectors an HPLC assay may be developed for the estimation of such low level actinomycin concentration.

VINCA ALKALOIDS

Important antineoplastic antibiotics are obtained from the plant Catharanthus roseus (Vinca rosea). Two members of this family of alkaloid-type antibiotics, vinblastine and rincristine, are used clinically[14]. Both the estimation of the useful vinca compounds in the different plant extracts and the separation and identification of metabolites require reliable quantitative analytical methods. Such methods are required to estimate the individual alkaloids in the presence of structurally related compounds and in the presence of other unrelated materials. Methods which rely on thin layer chromatography, spectrophotometric or colorimetric measurements do not fulfill all these analytical requirements. With this background in mind Gorog et al.[15] developed a method for these alkaloids that includes an HPLC system which can separate 26 related vinca alkaloids. The structures of all 26 compounds used in this study in relation to the basic structures are shown in Figure 1. The retention times obtained in the HPLC system are described below are also shown in Table 1. The chromatographic conditions were as follows: Instrument, Hewlett-Packard 1010 B; UV detector, operated at 298 nm; column, 250 cm x 4 mm, packed with Lichrosorb RP-8 (Merck, Darmstadt); solvent, acetonitrile-0.01 M ammonium carbonate (47:53); flow rate, 1.5 ml/min; injector, Valco loop, 25 µl.

In the process of assigning retention times to individual compounds of the alkaloid mixture, scanning was also done at

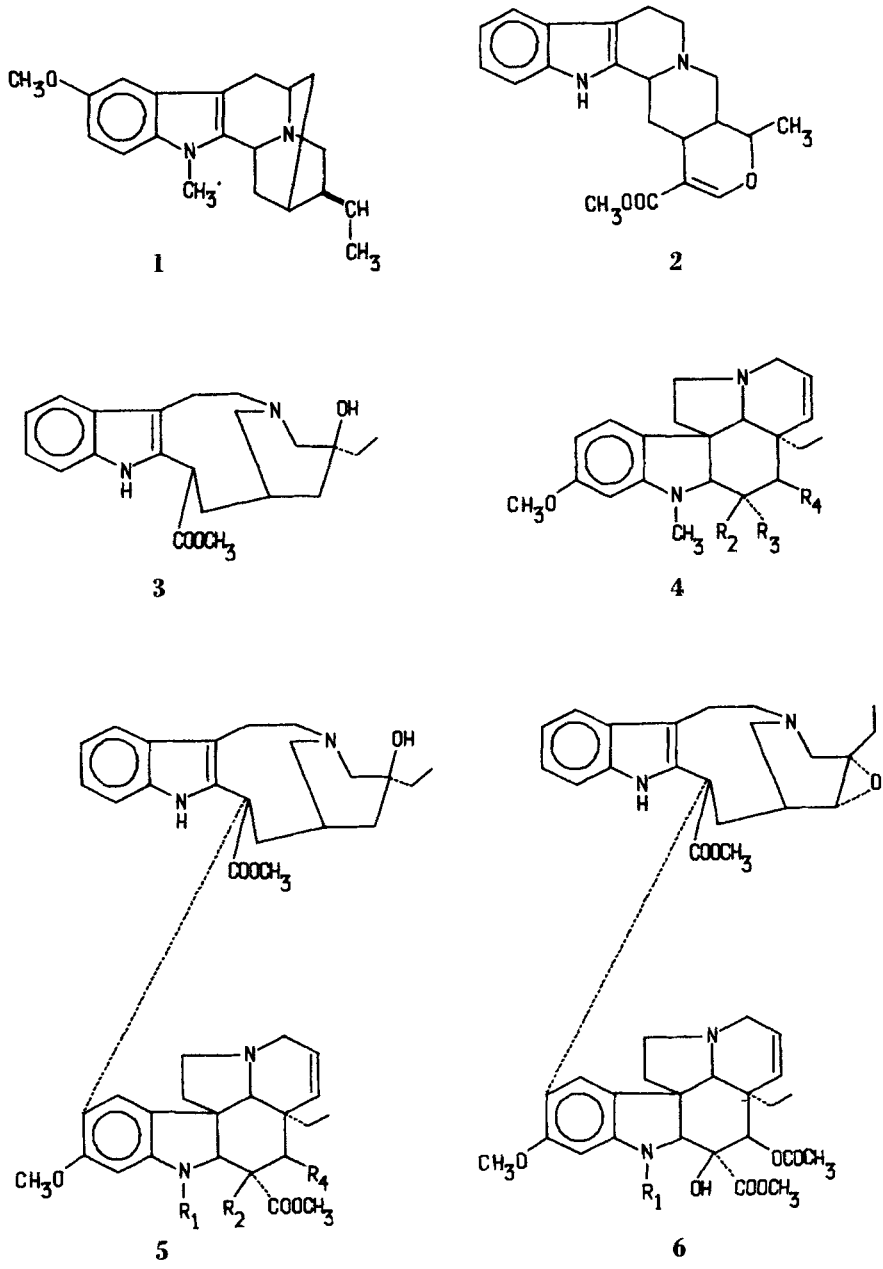


Figure 1. Structures of the vinca alkaloids studied by Gorog et al.(15) by HPLC.

Table 1. Retention data for vinca alkaloids [15]

| Compound | Retention time (min) | Structure[a] | Functional group[b] $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|---|---|
| Ajmalicine | 7.90 | 2 | | | | |
| Catharanthine | 9.75 | 3 | | | | |
| Desacetoxyvinblastine | 17.15 | 5 | G | J | | H |
| Formylleurosine | 9.75 | 6 | F | | | |
| Leurosine | 15.65 | 6 | G | | | |
| | 11.89 | 6 | H | | | |
| Lochnerine | 3.29 | 1 | | | | |
| Tetrahydroalstonine | 14.65 | 2 | | | | |
| Vinblastine | 12.37 | 5 | G | J | | B |
| | 7.04 | 5 | G | J | | J |
| | 12.52 | 5 | G | J | | C |
| | 10.25 | 5 | G | J | | D |
| | 10.09 | 5 | G | J | | E |
| | 48.10 | 5 | G | B | | B |
| | 6.18 | 5 | H | J | | J |
| | 10.04 | 5 | H | J | | B |
| Vincristine | 7.22 | 5 | F | J | | B |
| | 4.87 | 5 | F | J | | J |
| Vindoline | 6.16 | 4 | | J | A | B |
| | 4.71 | 4 | | J | A | J |
| | 9.39 | 4 | | J | A | C |
| | 5.84 | 4 | | J | A | D |
| | 4.30 | 4 | | J | A | E |
| | 23.22 | 4 | | B | A | B |
| Vindolinol | 4.44 | 4 | | J | K | J |
| Vindorosine[c] | 7.90 | | | | | |

[a] From Fig. 1.

[b] A=$COOCH_3$; B=$OCOCH_3$; C=$OCOCH_2Cl$; D=$OCOCH_2N(CH_3)_2$; E=$OCOCH_2NH(CH_3)$; F=CHO; G=$CH_3$; H=H; J=OH; K=$CH_2OH$.

[c] Vindoline without the aromatic methoxy group.

lower wavelengths so that structural assignments could be made from UV data obtained at two wavelengths. Although some of today's equipment (e.g., Waters Associates Model 440 UV detector) can be operated at two wavelengths simultaneously, Gorog et al. had to perform these operations in succession.

Much later, in 1981, Verzele et al.[16] published an HPLC system with gradient elution for the separation of vinca alkaloids. The specific objective of these authors was to assess the composition of crude Vinca rosea plant extracts. The crude solvent extract of a plant was extracted with an acid-water mixture and then subjected to chromatography. The following conditions were used: Instrument, Varian LC-5020; UV detector, operated at 280 nm, Varichrom; column, LiChroma filled with 10 μm RSiL-$C_{18}$-HL-D octadecyl silica gel; precolumn, 10 cm, filled with 20 μm RSiL or 20 μm RSiL-$C_{18}$-HL-D or with a mixture of the two to prevent dissolution of column material by the solvent; solvent, water-methanol with a gradient in methanol 50 to 85% (both solvents contained 0.1% ethanolamine; no gradient time was specified); flow rate, 2 ml/min; injector, Valco 7000 psi loop injector.

The resolution of this gradient technique is not much superior to that obtained by Gorog et al. by their isocratic system described above. However, by interruption of this gradient system the dimeric vinca alkaloids could be separated into two groups and the separation of some individual components

could be achieved better this way. This interrupted gradient technique seems to be a good method to fractionate complicated mixtures even at large scale.

Verzele et al. used the above HPLC technique to distinguish between monomeric and dimeric alcohols based on plate number (N) calculations. The authors observed that the required number of plates for separation of the dimeric alkaloids is smaller than that for separation of the monomeric alkaloids. To ascertain that this observation is correct for all temperatures the required N was determined between 5 and 50°C for each compound. The authors observed that changing the temperature not only changes N by a factor of 2 for monomerics and by a factor of 2.5 for dimerics, but simultaneously changes the capacity factor k' also. However, the average ratio of N between monomeric and dimeric alkaloids did not change greatly with changing temperature. To be certain that N values were not influenced by temperature changes, which would result in k' value changes, N value measurements were made at different temperatures at constant k' values by using different composition of eluants.

Measurements made with similar k' values indicated that the average N value ratios of monomeric and dimeric alkaloids do not change greatly with the temperature. From these studies the authors concluded that N values are indicative for structures of vinca alkaloids independently of the temperature of the

chromatography, and that the diffusion rates of these different alkaloids change parallel with temperature. They have also concluded that to obtain consistent k' values for identification purposes the temperature of the chromatography has to be closely controlled.

HPLC was also used to assess the composition of different plant extracts of vinca alkaloids in conjunction with radioimmunoassay[17]. HPLC served as the fractionation method and the fractions thus obtained were subjected to radioimmunoassay for identification of the individual vinca alkaloids. Conditions of the HPLC system were as follows: Instrument, Waters Associates, Model AC202; UV detector, operated at 254 μm; column, two reversed phase μBondapak $C_{18}$ (3.9 mm x 30 cm), connected in series; solvent, acetonitrile-0.01 M $Na_2HPO_4$ pH 7.4 buffer (1:1); flow rate, 10 ml/min; injector, Model U6K. The extract injected into the above system was prepared by a process that resulted in an acetonitrile solution of a material to be separated further by HPLC. The extraction process assured that all bioactive alkaloids were retained and that most of the materials harmful for the HPLC column were eliminated.

Besides estimation of composition of extracts or synthetic products of vinca alkaloids HPLC was also shown to be useful in the separation and identification of metabolites of these vinca-type antitumor antibiotics[18]. In this study

radiolabeled vinca alkaloids were injected into rats, and tissue samples were taken after certain time periods. These samples were prepared for HPLC injection by extraction-purification procedures. The HPLC system applied here was very similar to that discussed above[17] except that a gradient technique was used. Instrument, Waters Associates, Model 202; UV detector, operated at 254 μm; column, μBondapak $C_{18}$; solvent, acetonitrile-0.001 M $K_2HPO_4$, pH 7.5 buffer, linear gradient 20 to 80% acetonitrile, program time 40 min; flow rate, 2.5 ml/min. With this system vincristine has a retention time of 25 min. Fractions were collected for identification by taking eluates corresponding to UV-absorbing materials. Since the injected vinca alkaloids were radiolabeled the collected HPLC fractions could be checked for metabolites by scintillation counting technique.



## NEOCARCINOSTATIN

Neocarcinostatin is a chromo-protein [19] with considerable antitumor activity[20]. Its mode of action is connected to DNA strand scission[21]. Recently the chromophor portion of this antibiotic was separated from the apo-protein portion and was shown to possess all DNA-related biological activity previously attributed to the original antibiotic[22]. Separation of the chromophor and its different forms was achieved by HPLC. The intact antibiotic was extracted with 0.1 M acetic acid in methanol or 0.1 M HCl in

methanol or glacial acetic acid, in each case with an antibiotic concentration of 1.4 mg/ml. This extract was injected directly (50 µl) into the following HPLC system: Instrument, Waters Associates Model A2 C/GPC-204; detectors, UV, operated at 254 nm and a Schoeffel Model SF 970 fluorescence detector with 340 nm excitation and 418 µm emission cutoff filter; column, µBondapak $C_{18}$; solvent, 56 to 84% methanol in 0.01 M ammonium acetate, pH 4, concave gradient run for 50 min period; flow rate, 2 ml/min.



Fractions were collected by the above method and were analyzed for their relative DNA scission activity. Three UV active peaks, associated with biological activity were identified as the major chromophor and its chemical derivatives which formed during the hydrolysis of the antibiotic.

The above HPLC process could be extended for the collection of larger quantities of the chromophor[23]. For this purpose 500 ml methanolic solution, equivalent of 6.5 mg neocarcinostatin chromophor, was injected. The mobile phase was modified and consisted of 35 to 100% methanol containing 0.1% triethanolamine and 0.1% acetic acid in the solvent of 20% aqueous methanol containing 0.1% triethanolamine and 0.1% acetic acid delivered with a concave gradient program.

## BLEOMYCINS

Bleomycins are a family of glycopeptide antibiotics[24] and are used effectively against various human neoplasms,

especially against squamous cell carcinoma and sarcoma[25]. Pharmaceutical preparations used in treatment of these neoplasms contain several members of the bleomycin family of antibiotics. Because of the different toxicity of these different bleomycins the composition of the clinically used preparations must be controlled. To assess the exact composition of the preparations, various analytical methods were developed. Until recently the accepted method was based on a lengthy CM-Sephadex column chromatographic procedure[26]. This method has been replaced by a much faster and more precise HPLC method[27] which is able to separate 10 components of the clinically used bleomycin preparation. The method proved to be suitable for quantitation of most of these components. Some analytical values obtained with this HPLC method are shown in Table 2, in comparison with the CM-Sephadex column chromatographic method. A typical separation profile of the bleomycins is shown in Figure 2. The chromatographic conditions were as follow: Instrument, Waters Associates Model 6000, equipped with a valve type injector and constant flow pump; UV detector, Model 440, operated at 254 μm; column, μBondapak $C_{18}$ or Chromegabond MC-18; solvent, methanol-0.005 M 1-pentanesulfonic acid in 0.5% acetic acid, pH 4.3, linear gradient in methanol 10 to 40%, gradient mixing time 60 min; chromatographic time, 75 min; flow rate, 1.2-1.8 ml/min.

A previous attempt to analyze pharmaceutical preparations of bleomycin was described by T. T. Sakai[28]. This HPLC system

Table 2. Comparison of analyses of bleomycin samples by HPLC method[27] and by Sephadex column chromatography (CFR method)[26]

| | Bleomycin A, % in sample | | | Bleomycin B, % in sample | | |
|---|---|---|---|---|---|---|
| Sample | CFR Method | HPLC Method | SD | CFR Method | HPLC Method | SD |
| 1 | 68.43 (64.15)[a] | 62.30 | 0.46 | 28.33 | 28.10 | 0.80 |
| 2 | 67.60 (63.82)[a] | 63.16 | 1.12 | 28.54 | 29.49 | 1.25 |
| 3 | 66.52 | 59.50 | 1.26 | 30.85 | 28.48 | 0.77 |
| 4 | 65.78 | 60.33 | 0.85 | 31.39 | 29.89 | 0.66 |
| 5 | 69.27 | 65.12 | -- | 26.63 | 29.30 | -- |
| Av. SD | | | 0.92 | | | 0.87 |

[a]Calculation after HPLC separation.

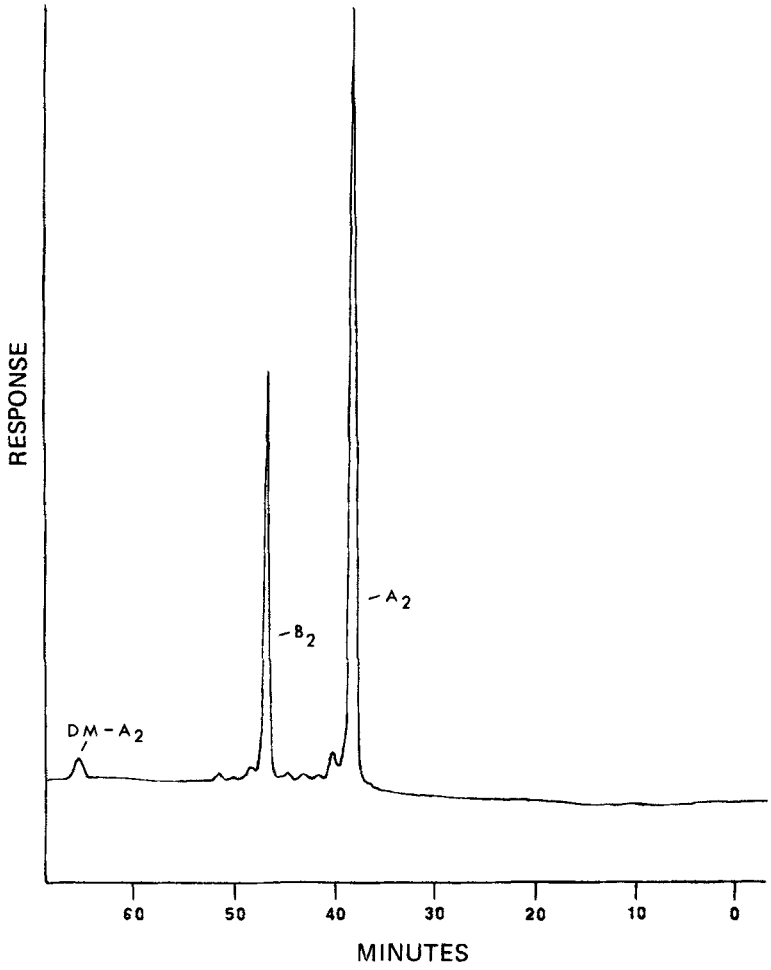


Figure 2. Separation profile of a commercial bleomycin preparation as performed by the method of Aszalos et al.(27).

could not resolve the minor components of such preparations.

The described HPLC system was as follows: Instrument, Waters Associates Liquid Chromatograph Model ALC-202/6000; UV detector, Model 440, operated at 254 or 280 nm and a variable wavelength absorbance detector; column, μBondapak $C_{18}$; solvent, 5 mM

1-heptane sulfonic acid in 50% aqueous methanol, pH 8.3; flow rate, 0.7 ml/min (1800 psi). This system provided an inferior chromatographic profile to that shown at Figure 1.

Vos et al.[29] used HPLC for the analysis of synthetic products of bleomycins. These authors prepared different demethyl bleomycin $A_2$ cobalt complexes. Products of individual reactions were analyzed for comparative yields by HPLC, and were also separated for chemical analyses by this technique. Experimental conditions given in the literature were as follows: Column, Nucleosil $C_{18}$ (Chrompack); solvent, 1% ammonium acetate-methanol (6:4); flow rate, 2 ml/min; injected amount, 1 mg.



Another previous HPLC method designed to isolate individual components of the bleomycin family of antibiotics was described by Rzeszotarski et al.[30]. This described system was used to separate bleomycin $A_1$, $B_2$ and $A_2$ components. The separation time was about 4.5 hours and a flow rate gradient was employed to accelerate the elution of the last component, bleomycin $A_2$, from the column. The bleomycins could also be analyzed in biological fluids, e.g. in urine[31]. To separate interfering materials present in urine, a 1.0 ml urine sample containing bleomycin was passed through a Sep-Pak $C_{18}$ cartridge and the cartridge was washed successively with water, acetone, water and methanol. The bleomycins were then eluted with 2 ml of 0.02 M sodium heptane sulfonate in methanol.

Aliquots of this eluate were injected into the analytical HPLC column. Chromatographic conditions were as follows: Instrument, Waters Associates Model M-6000A, with a loop type injector (Model U6K; UV detector (Model 440) operated at 254 μm; column, μBondapak $C_{18}$; guard column (3.9 x 60 mm), filled with pellicular reversed-phase packing of CO:Pell ODS; solvent, methanol-acetonitrile-0.0085 M sodium heptane sulfonate-acetic acid (30:10:59:1); flow rate, 2.0 ml/min (2500 psi). The recovery of bleomycin $A_2$ and $B_2$ from spiked urine samples was very good, with an average coefficient of variation of 7.4% and a relative error of 5.6%. The recovery from the cartridge was, at maximum, 85% if the cartridge was reconditioned with 0.02 M sodium heptane sulfonate in methanol.



## MITOMYCIN

Mitomycins are produced by a number of strains of streptomycin[32] and one member of this antibiotic family, mitomycin C, is of clinical interest as an antineoplastic agent[33]. Different chromatographic systems were developed for separation of the members of this antibiotic family, for quantitation of mitomycin C in biological fluids, for studying its mode of action, and for its quantitative determination in different pharmaceutical preparations.

Mitomycins and their chemical conversion products were successfully separated by HPLC technique in the course of

studying their interaction with nucleophiles in aqueous medium[34]. The following chromatographic conditions were used: Instrument, Waters Associates Model ALC/GPC 242, equipped with a Model 660 solvent flow programmer; UV detector, Model 202, operated at 245 μm; columns, Corasil II (61 cm x 2 mm) and μPorasil (30 cu x 4 mm); solvent, chloroform-methanol (9:1); flow rate, 1 ml/min (100 psi). For preparative work the μPorasil column was used with the solvent chloroform-methanol (92:8).



With the above analytical system, mitomycin A eluted at 2 min, mitomycin B at 10.5 min, mitomycin C at 18.3 min and sodium-7-amino-mitosane-9a-sodium sulfonate at 40 min. Several reduced mitomycin derivatives could be collected for analysis from the preparative HPLC system. The retention time of these compounds correlated well with their polarity, indicating the presence of an ideal distribution system.

A quantitative HPLC assay was worked out for the determination of mitomycin C in serum by Kono et al.[35]. Samples were prepared by ethyl acetate extraction of the serum, urine or ascites, followed by evaporation to dryness and injection into the chromatograph in methanol solution. Standard curves were prepared by using spiked body fluid samples. A linear relation was obtained between 1 and 25 μg mitomycin injected and the detector response. Chromatographic conditions were as follows: Instrument, Waters Associates Model 204; UV

detector, Model 440, operated at 365 nm; column, μBondapak $C_{18}$ (8-10 μm particle size), 300 x 3.8 mm; solvent, methanol-water (35-65); flow rate, 1 ml/min (1800 psi). The retention time for mitomycin C was 7 min. Using this sytem the authors could determine mitomycin C, at concentrations as low as 40 μg/ml concentration in biological fluids and could follow the concentration of this compound in serum of treated patients.

An excellent biochemical work establishing the mode of action of mitomycin C was published by Tomasz and Lipman[36], who used an HPLC method to separate nanomole quantities of the different metabolites and derivatives of mitomycin C[37]. Conditions were as follows: Instrument, Waters Associates Model 204; UV detector, operated at 254 μm; column, Ultrasphere-ODX (Beckman) 10 x 250 mm (semi-preparative); solvent, acetonitrile-0.03 M potassium phosphate (12.5:87.5), pH 6.0; flow rate, 2 ml/min (1100 psi). The separation profile of these compounds is shown in Figure 3.

Two metabolism studies of mitomycin C also used HPLC separation methods[38, 39]. These studies employed the following HPLC conditions: UV detector, operated at 365 and 313 nm; column, 100 x 3 mm $C_{18}$ (10 μm) radial compression cartridge filled with a 70 x 21 mm guard column packed with CO:Pell ODS; solvent, linear gradient of 0 to 50% methanol in 0.01 M phosphate, pH 7.0, gradient time 13 min; flow rate, 3

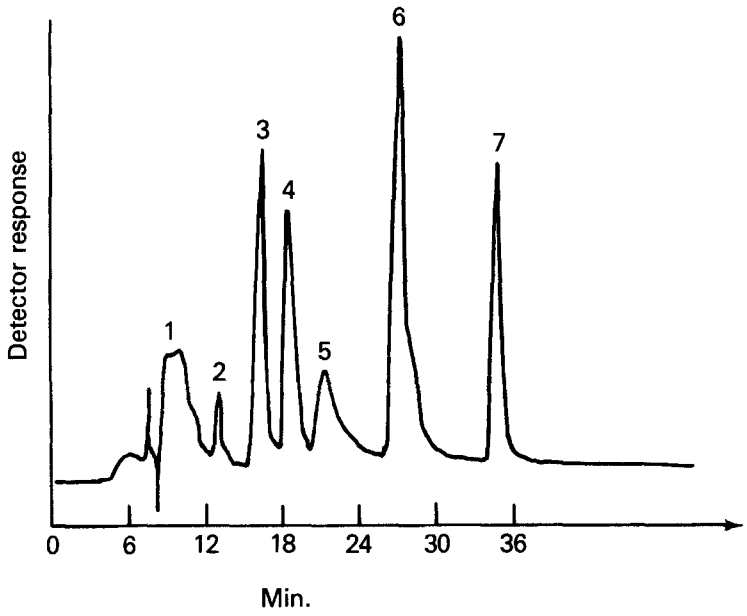


Figure 3. Separation profile of mitomycin and its metabolite by the method of Tomasz and Lipman(37). 1: Mixture of 1,2-cis- and 1,2-trans-2,7-diaminomitosene 1-phosphate; 2: 1,2-trans-1-hydroxy-2,7-diamino-10-decarbamoyl mitosene; 3: 1,2-cis-1-hydroxy-2,7-diamino-10-decarbamoyl mitosene and 1,2-trans-1-hydroxy-2,7-diamino-mitosene; 4: 2,7-diamino-10-decarbamoyl mitosene; 5: 1,2-cis-1-hydroxy-2,7-diaminomitosene; 6: 2,7-diamino-mitosene and 10-decarbamoyl mitomycin C; 7, mitomycin C. Quantities are in the 1-10 nanomole range.

ml/min. With this technique 9 mitomycin derivatives and mitomycin C could be resolved.

For the purpose of quantitatively assaying the mitomycin C content of pharmaceutical preparations an HPLC assay was developed by Aszalos et al.[40]. Bulk mitomycin C preparations were dissolved to a concentration of 0.4 mg/ml. Injectable samples were dissolved in water and extracted with

ethyl acetate, the ethyl acetate solution was taken to dryness and the residue was dissolved in methanol. From these methanol solutions of mitomycin C, 1 to 8 ng quantities were injected into the chromatograph and the areas under the peak, obtained by a microprocessor, were compared to those obtained with standard mitomycin C solutions. The assay has a standard deviation of less than 1%. Chromatographic conditions were as follows: Instrument, Waters Associates Model 6000 equipped with valve type injector; UV detector, Model 440, operated at 254 nm; column, μBondapak $C_{18}$; solvent, methanol-water (35-65); flow rate, 1 ml/min (1200 psi). This HPLC system is designed to replace the present microbiological method which was adopted for official use earlier.

A detailed paper by Tjaden et al.[41] deals with the comparison of several normal phase and reversed phase HPLC systems for the use of pharmacokinetic studies of mitomycin C. The compound was detected by ultraviolet and polarographic detectors and a simple procedure for its isolation from plasma, serum and urine was described. This isolation process was based on adsorption of mitomycin C on Amberlite XAD-2 resin and elution by methanol after a water wash of the resin. The dried sample was dissolved in mobile phase; porfiromycin (1 μg/ml) was added as internal standard, and the solution was injected into the chromatograph. Chromatographic conditions were as follows: Instruments, Waters Associates 6000A and Spectra Physics Model

740B; injector Model U6K and Model 7125 (Rheodyne); detectors, variable wavelength detector, LC-UV3, Pye-Unicam operated at 360 nm and polarographic detection system, PAR-310 (EG and G Instrument) cell, polarograph Model E100 (Bruker) hanging mercury drop electrode, 600 mV versus the silver/silver chloride reference electrode; columns, stainless steel, 3 x 100 mm, filled with Hypersil MOS as silica SI-60 (63-200 μm); solvents, ethyl acetate-methanol-water-dichloromethane (97:2:1:1) for normal phase and 10-12% acetonitrile in 0.05 M phospate buffer, pH 7.0, or 10% acetonitrile in water for the reversed phase column; flow rate, 0.5 to 2.0 ml/min.

The sensitivity of the above system was 5 mg/ml. The normal phase column was found more stable, less expensive and more suitable for electrochemical detection, and was used for detecting mitomycin C metabolites. The reversed phase system was used for rutin analysis.



## STREPTOZOTOCIN

Streptozotocin, 1-methyl-1-nitrosourea-2-deoxyglucose, shows promise in the treatment of pancreatic islet cell carcinoma[42]. During the clinical trials of this drug, the amount of unchanged streptozotocin in serum had to be assessed[43]. One of the analytical methods used for this purpose was HPLC. In this method identification of the streptozotocin peak was based on specific colorimetric analysis

of the eluted materials, $^{3}H$ and $^{14}C$-labeled streptozotocin, and UV analysis. Performance of the HPLC system was checked each time by measuring retention time of a drug standard ($t_R$ = 17 min). Samples were prepared for analysis as follows: plasma proteins were removed by ethanol precipitation, the supernatant was adjusted to pH 4, and urine was lyophilized, taken up in methanol-acetone (3:1) and centrifuged. From the plasma or urine samples prepared in this manner, 10 µl was injected into the high pressure liquid chromatograph. Conditions were as follows: Instrument, not described; UV detector operated at 254 nm; column, 3,3'-oxypropionylnitrile (Durapak); solvent, hexane-isopropanol (3:1). The above system gave a linear relation from injected amount of streptozotocin and detector response from 3 to 90 µg/ml.



## RIFAMYCINS

Some rifamycin-type antibiotics are reverse transcriptase inhibitors and were implicated in cancer chemotherapy. Below are given some examples of the use of HPLC in the determination of rifamycin and its metabolites in human plasma and in separation of chemical conversion products of rifampicin.

One method described quantitation of rifamycin and its main metabolite, 25-desacetylrifampicin, in plasma by an HPLC system with a silica gel column[44]. An extension of this method described by Lecaillon et al.[45], who were using the same

technique, achieved the same level of detection (0.1 μg/ml), but could also simultaneously assay another metabolite, 3-formyl rifamycin SV. Conditions of this HPLC method were as follows: Instrument, Hewlett-Packard, Model 1011, equipped with Waters Associates U6K valve injector; UV detector, Hewlett-Packard Model 1036, operated at 254 nm; column, 100 x 7.5 mm stainless steel column filled with LiChrosorb Si 60 (5 μm), by a special filling technique. The column efficiency was estimated to be 2000-3000 theoretical plates for the third peak appearing around 8 min elution time; solvent, dichloromethane-isooctane-ethanol-water-acetic acid (36.6:45:16.8:1.65:0.002); flow rate, 3 ml/min. For HPLC analysis, rifamycin and its metabolites were extracted from plasma, urine or saliva and the quantities injected (5-200 μl) were selected according to the approximate concentration of the drug in these body fluids. Precision of the measurements was ± 5%. Calibrations with all four compounds were done every day.

It was believed that this technique would be improved by introducing a reversed phase column for the analysis of the above compounds and an additional metabolite, N-desmethyl rifampicin[46]. Good baseline separation could be obtained for most of the compounds except for rifamycin and N-desmethyl rifamycin. Therefore the system was used to quantitate only rifamycin and 25-desacetylrifamycin. Two chromatographic conditions were used. Conditions of the first system:

Instrument, Hewlett-Packard Model 1084B; UV detector, Model 79870A, operated at 254 nm; column, PP-8, 10 μm, 250 x 4.6 mm, Brownlee Labs; solvent, 0.1 M $KH_2PO_4$, pH 3.5 with 0.2 M $H_3PO_4$-acetonitrile (62:38); flow rate, 2 ml/min; temperature, 30°C. Conditions of the second system: Instrument, Waters Associates 6000A; UV detector Model 440, operated at 254 nm; column and flow rate as in the first system; solvent, 0.1 M $KH_2PO_4$, pH 3.5, 0.2 M $H_3PO_4$-acetonitrile-water (60:36:4); temperature, 20°C. With the second instrument a Waters Associates W.I.S.P. Model 710A sample programmer was used for rutin analysis. The standard deviations of these determinations were between 3 and 5.8% depending on the compound and its concentration. Limit of detection was 0.2 mg/ml.

An interesting HPLC system was developed for the separation of rifamycin and its metabolites from other drugs, using a single column and sequential elution with different solvents[47]. Depending on which of the solvent was used first, the different drugs present on the column could be eluted and quantitated. With one of the solvent systems, (A), 4,4'-diaminodiphenyl sulfone and its metabolites could be analyzed; with the second solvent, (B), rifamycin and clofazimine could be analyzed simultaneously; with a third solvent, (C), clofazimine could be quantitated alone. Chromatographic conditions were as follows: Instrument, Waters

Associates Model 6000A, with injector Model U6K; UV detection, Jesco Uridec 100 II spectrophotometer; column, μBondapak $C_{18}$; solvent A, acetonitrile-water (20:8); solvent B, tetrahydrofuran-0.5% acetic acid (40:60); solvent C, tetrahydrofuran-water (50:50, containing 0.0025 M 1-pentanesulfonic acid); flow rate, 2.0 ml/min with solvent system A, 1.5 ml/min with solvent systems B and C. The temperature was controlled in each case at 20 ± 2°C. After solvent A was used for the analysis of the different sulfones, solvent B could be introduced into the system directly. However, solvent B could be followed by solvent A only if methanol water (1:1) was pumped through the system after solvent B. All chromatographic profiles shown in this detailed paper indicate good baseline separation.

With the aim of controlling the hydrogenation reaction of rifamycin an HPLC system was developed by Vlasakova et al.[48]. The system proved to be useful to quantitatively assess rifamycin, the quinone form of rifamycin and their dihydro and tetrahydro derivations. Chromatographic conditions were as follows: Instrument, Varian Model 4100; UV detector, Variscan UV spectrophotometer (Varian), 8 μl cell, operated at 334 nm; column, 250 x 2 mm, filled with Micro Pak $NH_2$ (10 μm); solvent, chloroform-methanol (97:3); flow rate, 0.2-0.7 ml/min. Two other columns tried for the above purpose were found not to be suitable: Micro Pak Si-10 and Pmicro Pak CN

with chemically bonded alkyl nitrile groups. During this study capacity factor and theoretical plate height (H) calculations were made. The formula for the calculation of H was $H=(L/16)(W_{t/t_R})^2$ where L is the column length and $W_t$ the peak width at the baseline. Optimal plate heights were 0.5-1.9 mm.

## ANTHRACYCLINE ANTIBIOTICS

The most important members of this antibiotic family are the clinically useful adriamycin (doxorubicin)[49] and daunorubicin (daunomycin)[50]. Most HPLC studies were therefore done with these two antibiotics, their degradation products and metabolites. A large number of papers have been published on HPLC analysis of these drugs. They can be divided into four groups: quantitation in pharmaceutical preparations, quantitation in body fluids, metabolite studies and study of chromatographic conditions of these drugs.

The official method accepted by the Food and Drug Administration for determination of adriamycin in bulk pharmaceutical preparations is described in the Code of Federal Regulations[51]. The method is as follows: Instrument, Waters Associates Model 244 (or equivalent); UV detector, operated at 254 nm; column, μBondapak $C_{18}$; solvent, water-acetonitrile (69:31), adjusted to pH 2.0 with phosphoric acid; flow rate, 1.5 ml/min; internal standard, 2 mg/ml solution of 2-naphthalene sulfuric acid in the solvent mixture.

This HPLC system was not designed to determine adriamycin and daunorubicin simultaneously. Also the possible impurities, the aglycones adriamycinone and daunorubicinone, could not be assayed. For these reasons Haneke et al.[52] designed an HPLC system in which all four of these compounds could be quantitated simultanenously. The system is not designed to use an internal standard but relies on standard curves obtained with each new chromatographic setup. Conditions were as follows: Instrument, Waters Associates 6001A; UV detector, Model 240, operated at 254 nm; injector, Model U6K, valve type; column, μBondapak $C_{18}$; solvents, best solvent methanol-0.1 M $NH_4H_2PO_4$ (65:35), pH 4.0, other useful solvent methanol-0.005 M 1-heptanesulfuric acid (62.5-37.5), pH 3.5; flow rate, 1 or 2 ml/min (1000 or 1600 psi). The aglycones could be estimated as low as 0.5 relative % in preparations by this method. Area under the peak was calculated by a microprocessor (integrator 3380A, Hewlett-Packard).

Good separation could be obtained for adriamycin, daunorubicin and a third anthracycline antibiotic, carminomycin, when acetonitrile-0.025 M camphorsulfuric acid, pH 3.8, was used as solvent with a column of 5 μm $C_8$ Lichrosorb[53].

An assay was worked out for anthracycline concentration in formulated drug products by Averbuch et al.[54], using an aminocyanosilica column. The sensitivity of the assay was about 5 ng/ml. Six anthracycline antitumor agents, all from the

bohemic acid complex, were isolated from fermentation broth by preparative HPLC using a normal phase column[55]. HPLC was shown to be useful in determining stability of anthracyclines in infusion fluids[56]. One other study aimed to determine adriamycin in pharmaceutical preparations was presented by Barth and Conner[57]. A method based on electrochemical detection, applying 0.65 V oxidative potential, to monitor daunorubicin and its metabolite in different body fluids was designed by Akpofure et al.[58], and compared with fluorescence detection. It was found that the electrochemical detection was more sensitive than fluorescence detection and that a sensitivity of 10 ng/ml of compound could be achieved.

Adriamycin and daunorubicin are determined in fermentation broth at different fermentation times in order to determine maximum production yield. For this purpose Alemanni et al.[59] extracted the broth at pH 1.5, and analyzed the extract on a μBondapak $C_{18}$ column using acetonitrile-$KH_2PO_4$ buffer, pH 3 (citric acid), (7:18) and UV detection at 254 nm. Similar systems were developed by Stroshane et al.[60] using direct injection of the acidified, heated and filtered fermentation broth.

For assaying daunorubicin hydrochloride content in different forms of pharmaceutical preparations the U. S. Pharmacopeia adopted the following HPLC method.[61] Samples are dissolved in a 2 mg/ml solution of 2-naphthalene sulfuric acid containing

a mixture of water-acetonitrile (62:38), adjusted to pH 2.2 with phosphoric acid. Other conditions are as follows: Column, μBondapak $C_{18}$ or equivalent column; detector, UV, operated at 254 nm; solvent, water-acetonitrile (62:38), pH 2.2 (phosphoric acid); flow rate, 1.5 ml/min; resolution factor between daunorubicin and 2 naphthalene sulfuric acid, 2.0; calculation of potency as μg daunorubicin per mg: (25 C/W) (Ru/Rs) where C is the concentration in μg in the standard preparation, W is the weight in mg of daunorubicin hydrochloride and Ru and Rs are the ratios of peak responses of daunorubicin peak to 2-naphthalene sulfuric acid peak obtained with the unknown preparation and the standard preparation, respectively.



An efficient extraction and separation method was worked out by Pandey and Toussaint[62] for the detection of different anthracyclines in fermentation broth. The new extraction method relied on extraction of the mycelium at pH 1.5 into aqueous solution. After this aqueous layer is filtered, it is applied directly to the high pressure chromatographic system. The HPLC system was optimized to separate and quantitate several components of this type of fermentation: daunorubicin, baumycin $A_2$, daunorubicinone, 7-deoxydihydrodaunorubicinone, E-rhodomycinone and an unknown structure 30-8-1M. The optimized HPLC system was as follows: Instrument, Waters Associates 6000A, with Model 660 solvent programmer and U6K universal injector; detector, Schoeffel SF 770 Spectroflow, operated at

254 nm; column, μBondapak $C_{18}$; solvent, water (pH 2.0, $H_3PO_4$)-methanol (35-65 or 40-60), solvents filtered separately and mixed afterwards; flow rate, 2 ml/min. A typical separation profile is shown in Figure 4.

The determination of anthracycline antibiotics in body fluids was described in several papers. One of the earliest papers was that by Hulhoven and Desager[63] who used a quaternary solvent system and adriamycin as internal standard to estimate daunorubicin in plasma. Later Pierce and Jatlow[64] provided an assay for adriamycin and adriamycinol and the two aglycones in human plasma. For internal standard these authors used daunorubicin and daunorubicinone (2 μg/ml each, in methanol). Samples were mixed with internal standards, made alkaline and extracted with five volumes of chloroform-isopropanol (2:1). After separation of the phases the organic phase was taken to dryness and the residue was dissolved in methanol. From this solution an aliquot was injected into the chromatograph. Chromatographic conditions were as follows: Instrument, Perkin-Elmer dual pump Model 601; fluorescence detector, Perkin-Elmer Model 204-S, or Model 650-10LC, operated at 465 nm excitation and 580 nm emission wavelength; column, ODS "Hi-Eff" $C_{18}$, 5 μm, reversed phase; solvent, acetonitrile-0.01 M phosphoric acid, pH 2.3 (40:60) (for measurements of the aglycones the solvent was acetonitrile-0.01 M phosphoric acid (36-40:64-60)); flow rate, 1 ml/min; temperature, 25°C.

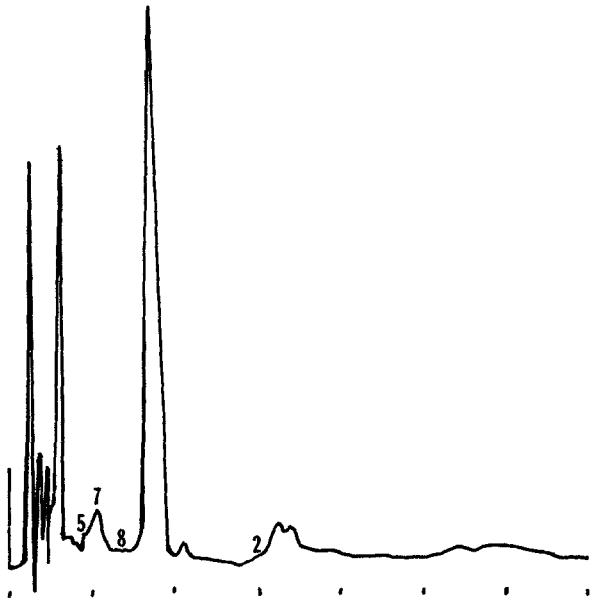


Figure 4. Separation profile of extracted anthracyclines from fermentation broth as described by Pandey and Toussaint(62). 2: daunorubicin; 5: daunorubicinone; 7: 7-deoxydihydrodaunorubicinone; 8: E-rhodomycinone.



Quantitation was based on peak height ratios. In this system $t_R$ for daunorubicin was 18 min and adriamycin, its metabolite and two aglycones eluted before 18 min. It was shown that many other drugs, if also present in the plasma, would not interfere with this assay. Because of the use of the fluorescence detector the sensitivity of this determination could be increased to 2 ng/ml.

Eksborg et al. provided a similar determination for adriamycin and adriamycinol simultaneously(65). A LiChrosorb RP-2, 5 μm column and the same solvent system as in reference 54

was used. The sensitivity of their measurement was somewhat less than described in reference 54, perhaps because a slightly modified extraction process and different setting of the fluorescence detector (Schoeffel Instrument FS-970) were used.

Another HPLC assay system was aimed to determine adriamycin, adriamycinol, aclacinomycin A and two 4''' isomers of aclacinomycin A, MA-144-171 and MA-144-N1, in rabbit plasma[66]. The extraction of the plasma was very similar to those described in the above HPLC systems used in connection with assays of adriamycin. HPLC conditions were as follows: Instrument, not described; detector, spectrophotofluorometer, operated at 475 nm excitation and 580 nm emission wavelength; column, 5 μm LiChrosorb SI 60; solvent, chloroform-methanol-ammonium hydroxide-water (855:130:10:5) for adriamycin and adriamycinol and chloroform-dimethylsulfoxide-ethylene glycol (975:20:5) for the carminomycin-type compounds; flow rate, 1.1 ml/min; lower limits of sensitivity of the assay, 2-4 ng adriamycin/ml and 10-20 ng/ml aclacinomycin. It is interesting to note that the silica gel adsorption system gives almost equivalent sensitivity to the reversed phase as shown in the above two examples.

Tissue distribution of liposome-entrapped adriamycin was followed by HPLC[67]. A simple extraction followed by HPLC provides quantitation for daunorubicin and adriamycin in tissues[68]. Similarly HPLC assays were developed for

estimation of adriamycin and daunorubicin in urine[69] in lymph and gall[70], carminomycin in human plasma[71] and adriamycin in biological fluids[72].

Two other studies described the determination of daunorubicin and daunorubinicol in plasma of humans administered the parent compound[73] or its DNA complex[74].

Some other very good work should be mentioned here briefly. Brown et al.[75] determined daunorubicin and 7-con-o-methyl nagarol in plasma, using a fluoresence detector, a Pye LC-XPD pump and a LiChrosorb RP-2 (5 µm) column, capable of estimating daunorubicin at 5 µg/ml. Moro[76] et al. quantitated 4'-epiadriamycin and its 13-dihydro derivative in human plasma, also using a fluorescence detector, a Spectra-Physics Model SP 3500 B chromatograph, a Partisil ODS column and a CO:Pell ODS Whatman precolumn, 7 cm x 2.1 mm. S. E. Fandrich[77] developed a system to determine carminomycin in serum. In connection with this assay a very good extraction process developed, starting from human serum and allowing carminomycin and carminomycinol to be measured at 2 µg/ml.

An analytical HPLC system was devised to follow the fate of adriamycin in vivo[78]. This system involved two HPLC columns, one reversed phase and one normal phase. Drug and metabolites were determined in plasma, bile and urine. Sample preparation for HPLC was about the same as in the studies described above, except that a protein precipitation step

(methanol) was introduced before chloroform extraction of the anthracycline compounds at pH 8.5. The chromatographic system was as follows: Instrument, Waters Associates Model ALC/202 and Model ALC/244 for the reversed phase studies; fluorescence detector, Schoeffel Instrument Model SF-970, operated at wavelengths of 482 nm for excitation and 550 nm for emission; column, Partisil-10 PAC for normal phase and Bondapak phenyl for reversed phase; solvent, chloroform-methanol-acetic acid-water (850:150:50:15); initial convex gradient (profile α4) set for 2 min starting with 10% of the above solvent in chloroform and finishing with 100% of the above solvent system, the initial 2 min followed in 6 min with the final solvent to elute all compounds (normal phase system); linear gradient starting at 30% acetonitrile (containing 2% buffer) and 70% pH 4.0 ammonium formate buffer, finishing at 35% acetonitrile and 65% pH 4.0 ammonium formate buffer, gradient time 5 min (reversed phase); flow rate, normal phase 2 ml/min, reversed phase 3.5 ml/min. In each case the column was re-equilibrated with the starting solvent system. The peaks could be quantitated by peak height measurements with the reversed phase system but the "cut and weigh" method had to be employed with the tailing peaks of the normal phase system. Sensitivity of the reversed phase system was 3.0-0.5 μg/ml depending on the compound assayed. The use of the two chromatographic systems was thought to provide an unequivocal identification basis for adriamycin and its

metabolites. It should be mentioned here that compared to the other studies discussed above the design of this system is somewhat complicated, and equally good results were obtained by a single column isocratic solvent system described in the other studies.

Baurain et al.[79] studied the cellular uptake and metabolism of daunorubicin in L1210 leukemic cells and followed the events by HPLC. They were successful in determining concentrations at the level of 1.5 ng/ml. For internal standard adriamycin was used (10 μg/ml), from which 0.1 ml was added to a 2 ml cell suspension. Daunorubicin was extracted by chloroform-methanol (4:1) at pH 7.2 and the organic phase was injected into the chromatograph. The HPLC system was as follows: Instrument, Hewlett-Packard, Model 1084 with a 6-part injection valve, Rheodyne Model 7120, 20 μl loop; detector, Gilson Fl-1A/B fluorimeter, operated at 480 nm excitation and 560 nm emission wavelength; column, LiChrosorb Si 60; solvent, chloroform-methanol-acetic acid-water (720:210:35:30); flow rate, 1 ml/min. The system provided very good calibration curves for daunorubicin, daunorubicinol and the aglycone, with good reproducibility and very well-resolved peaks.

Many other pharmacological studies were conducted with HPLC, in addition to those described above. The HPLC methodologies of these studies are in large part similar to the ones detailed above and will not be quoted here. However, most of them are

listed below with the objective of the study. Metabolism of daunorubicin was studied in sensitive and resistant Ehrlich ascites tumor cells[80] and in rat liver microsomal preparation[81]. The pharmacokinetics of adriamycin were studied in Ehrlich tumor-bearing mice[82] and in gynecologic carcinoma patients[83]. The same type of study was made of 4'-epi-adriamycin in patients with impaired renal function[84] and for aclacinomycin A in cultured L1210 cells[85].

In an excellent paper Eksborg described optimization of the chromatographic conditions for adriamycin, daunorubicin and their 13-hydroxylated metabolites[86]. First the retention value of the compounds (expressed as log k') versus mobile phase composition (log (R), molar concentration of organic modifier) was studied. Using acetone, acetonitrile and ethanol as organic modifiers maxima were found for log k' between 0.8 log (R) and 1.1 log (R) for each modifier and for all four compounds studied. Next the selectivity was studied. For this purpose the log(R) was plotted against separation of the different compounds expressed as the log $\alpha$ ($\alpha$ is the separation factor relative to adriamycinol). Again for each solvent modifier very good separation could be obtained at relatively low concentrations of the studied compounds. Least separation was obtained with ethanol; other alcohols like methanol or propanol did not give any better results. A third factor, the length of the alkyl chain bound on the silica gel support, was studied in

relation to retention time and selectivity when acetonitrile was used as organic modifier. It was found that with increase of the length of the alkyl chain (RP-2, RP-8 and RP-18) the retention time of each component increases but no change in selectivity occurs.

It was deduced from the results of those experiments that the shortest separation time can be achieved according to the function

$$f(\alpha, k'_2) = (4R_s\,\alpha)^2(\alpha - 1)^{-2}(1 - k'_2)^3\,k'^{-2}_2$$

where $R_s$ is chromatographic resolution and $\alpha$ and $k'$ are as defined above. Further considerations yielded the necessary number of theoretical plates for the separation of each antibiotic from its 13-hydroxy derivative.

The chromatographic conditions of these experiments were as follows: Instrument, LDC-711 solvent delivery system pump; Rheodyne Model 70-10 injection valve with a 100 µl sample loop; detector, LDC Spectromonitor (8 µl) operated at 500 nm; column, one 150 and one 50 mm x 4 mm OD, filled with LiChrosorb RP-2, RP-8 or RP-18 (each 5 µm); solvents, 20 to 90% acetonitrile, acetone or ethanol in water; flow rate, 1.7 mm/sec.

## USE OF HPLC IN THE ISOLATION STUDIES OF NEWLY DISCOVERED ANTITUMOR ANTIBIOTICS

There are many examples in the literature of the use of HPLC for new antitumor antibiotics, and not all of them can be cited

here. HPLC apparently has proved to be an important tool in the isolation of newly discovered antibiotics. A few typical examples are given.

One example is the work dealing with the detection of the gilvocarcin antitumor antibiotic complex[87]. The original problem concerned a fermentation broth containing an unknown antibiotic complex. To assess the quantity in the broth of the two major components of this complex, 2064A and B, an HPLC system was developed. Conditions were as follows: Instrument, Waters Associates 6000A; UV detector, operated at 254 nm; column, μBondapak $C_{18}$; solvent, methanol-water (70:30); flow rate, 1.5 ml/min. The retention times obtained for the two major components suggested the presence of gilvocarcin type antibiotics in the unknown fermented complex. Other identification methods were then used which required more material, and a preparative HPLC method was developed for this purpose. The following conditions were used: Instrument, Waters Associates 6000A; UV detector, operated at 254 nm; column, $C_{18}$ Magnum semipreparative, 50 x 9.4 mm column (Whatman); solvent, methanol-water-tetrahydrofuran (40:45:15); flow rate, 5.0 ml/min.

Another example of the use of HPLC in isolation studies of antitumor antibiotics was described by Pandey et al.[88], in connection with the antibiotic fredericamycin. Very small quantities of this antibiotic complex were isolated by two

different methods from the fermentation broth. The most biologically active component in each complex was identified by HPLC studies. The $t_R$ values of the most active component, fredericamycin A, with μBondapak $C_{18}$ or μPoracil columns were 6.5 and 6.0, respectively. Conditions of the HPLC studies were as follows: Instrument, Waters Associates Model 6000A; UV detector, Schoeffel Model SF 770 variable wavelenth, operated at 254 nm; columns, μBondapak $C_{18}$ and μPorasil; solvent, methanol-water-acetic acid (70:30:1) or chloroform-methanol-acetic acid (87:3:3); flow rate, 2 ml/min or 1 ml/min. These HPLC systems also could be used to isolate small quantities of the antibiotic for biological assays.

When concentrations of individual components in an isolated antibiotic complex have to be assessed, HPLC can be a very useful technique, as shown by Argoudelis et al.[89]. This research team had to evaluate a number of fermentation broths for yield and composition of the paulomycin antibiotic complex. In this investigation, a Hewlett-Packard instrument was used to determine ultraviolet spectra of components producing individual peaks in the HPLC chromatogram. These spectra indicated that four components of the isolated complex belong to the same antibiotic type, the paulomycins. Operational conditions were as follows: Instrument, Hewlett-Packard Model 1084B, operated with the dual pump mode; UV detector, HP Model 79875A variable wavelength detector, operated at 320 nm; column, Brownlee 100 x

4.6 mm packed with 10 μm reversed phase filling; solvent, acetonitrile-0.5 M potassium phosphate buffer, pH 7.0 (38:62); flow rate, 2 ml/min; temperature 30°C.

Another example of the use of HPLC in the isolation studies of antitumor antibiotics can be cited in connection with the first isolation of gilvocarcins[90]. These antibiotics were difficult to separate because of solubility problems. The preparative HPLC method designed to solve this problem had the following details: Instrument, Waters Associates Prep LC/system 500; detector, variable wavelength Schoeffel Spectroflow SF 770, operated at 400 nm; column, Prep Pak (two) 500 silica gel column with radial pressure of 40 atm, partially deactivated by passing through 2.5 l of solvent (ethyl acetate-isopropanol-water (87:13:0.5)); flow rate, 100 ml/min; sample 100 to 300 mg crude antibiotic in 500 ml eluting solvent. The antibiotic complex was recirculated 8-10 times until individual members of the complex separated. The fractions finally obtained were assayed for homogeneity by an analytical HPLC system.

A family of new antibiotics active against Ehrlich carcinoma of mice was isolated by Anke et al.[91] and named the deflectins. The initial chromatographic methods used to isolate a crystalline product led the investigators to believe that they obtained a homogeneous antibiotic. However, high pressure chromatography indicated the presence of five major and several minor components in the original crystalline isolate. Since

different fermentations produced deflectin complexes of different compositions, the following HPLC system was developed to analyze the crude products of these fermentations: Instrument, Waters Associates 6000A; detector, Waters 450 and R401, operated at 339 nm; column, reversed phase silica (RP-18); solvent, methanol-triethylammonium formate buffer, pH 6.0 (8:2). No other details were given for this system. Also, the preparative HPLC method used to isolate the individual members of the deflectin antibiotic family was omitted.

Many antitumor antibiotics are isolated from plants. A variety of separation and purification methods, including HPLC, are used in these isolation studies. A good summary of the use of HPLC in these isolation studies was given by Wall et al.(92).

HPLC proved to be an excellent tool in assessing the concentration of the antibiotic produced in a fermentation broth at different times, which is important for the selection of the harvest time. Examples of this approach were described by Tsuji and Goetz(93) who used HPLC to monitor erythromycin and tetracycline fermentations. In another study Ogasawa et al.(94) followed the fermentation of aclacinomycin and related compounds by the organism Streptomycos galilaens. The key factor in this HPLC monitoring study was that the selection of the detector wavelength, 436 nm, put all the cofermented related compounds, aclacinomycin B, MA144-L1, -MI, -NI, -S1, -T1 and

-D1, on an equal molecular absorbance basis, and therefore on an equal quantitation basis by simple peak area determination. An internal standard was added to an aliquot of the fermentation broth to facilitate calculation of the recovery yield. The samples were prepared for HPLC by extraction of the broth with toluene at pH 7.5, centrifugation and filtration of the toluene layer. The chromatographic conditions were as follows: UV detection: 436 nm; column, μPorasil; solvent, chloroform-methanol-acetic acid-water-triethylamine (68:20:10:2:0.01, v/v); flow rate, 1.0 ml/min (1000 psi). The system provided baseline separation for six fermentation products with symmetrical peaks. Two new anthracycline antibiotics, auramycin and sulfurmycin, were isolated recently[95]. To show the novelty of these antibiotics, their sugar moiety was hydrolyzed and the isolated aglycones were compared to those of known anthracyclines by HPLC. The chromatographic conditions of this study were: UV detection, 430 nm; column, μBondapak $C_{18}$; solvent, methanol-water (60:40), with heptanesulfonic acid; flow rate, 1 ml/min. After showing differences in migration rates of these two new aglycones and those of known ones, the additional physico-chemical studies revealed that these new anthracyclines differ from known anthracyclines by a new combination of side chains on the aglycone moiety.

Five endogenous growth inhibitors isolated from JB-1 ascites tumor were purified by a combination of several methods,

including HPLC[96]. These growth inhibitors were small glycopeptides and were extracted from the tumor by methanol. The methanol-extracted material could be separated into five components by LH-20 column chromatography. The HPLC conditions for the final purification were as follows: Instrument, Hewlett-Packard 1084B and Waters Associates 6000A; detector, Cecil CE 588 UV Scanning Spectrophotometer operated at 280 nm and Model 440; column, Nucleosil 5 μm $C_{18}$ and Nucleosil 5 μm CN; solvents, water-acetic acid (96:4) or 0.1% trifluoroacetic acid in water for 2 min followed by a gradient of 2% acetonitrile per min up to 20% acetonitrile; flow rate, 0.5 ml/min or 1.0 ml/min; temperature, ambient or 40°C. These two HPLC conditions refer to the purification conditions of two growth inhibitors separated by the LH-20 column.

Cadequomycin, a novel nucleoside analog antibiotic, was isolated from fermentation broth by recycling preparative HPLC[97]. In another study[98] the chromophores could be isolated from two peptide-type antibiotics, macromomycin and auromomycin. Among other techniques, like UV spectroscopy and antibacterial spectra, HPLC was used to show that the two isolated chromophores are identical.

## USE OF HPLC IN METABOLIC STUDIES OF VARIOUS ANTITUMOR ANTIBIOTICS

The use of HPLC in metabolic studies was already detailed in the discussion of the above antibiotics. However, some studies

along these lines with other antibiotics are discussed as a special group for reasons outlined below.

One assay concerns the analysis of 1 hexylcarbamoyl-5-fluorouracyl (HCFU) and its metabolites, 5-fluorouracyl (FU), 1-w-carboxypentylcarbamoyl-3-fluorouracyl (CPEFU) and 1-w-carboxypropylcarbamoyl-5-fluorouracyl (CPRFU). While FU is therapeutically advantageous and important, CPRFU and CPEFU are connected with side effects. To provide an optimal drug administration schedule, the pharmacokinetic studies required a fast and sensitive monitoring assay. Such an assay was worked out by Kono et al.[99], utilizing HPLC. Specimen samples were prepared by homogenizing the tissue, centrifuging at 7000 g, acidifying with 1 N HCl, extracting with ethyl acetate, taking to dryness and dissolving the obtained residue in methanol. Chromatographic conditions were as follows: Instrument, Waters Associates liquid chromatograph equipped with Model 6000 solvent delivery system and Model U6K injector; UV detector, Model 440, operated at 254 nm; column, μBondapak $C_{18}$/Porasil (10 μm particle size); solvent, water-tetrahydrofuran-acetonitrile (50:35:15) for the quantitation of HCFU, water-tetrahydrofuran (65:35) for separation and quantitation of CPEFU and CPRFU; flow rate, 1 ml/min (1500 psi). The two-solvent system was necessary because in the first system the retention times of CPEFU and CPRFU were too close for quantitation.

While aclacinomycin belongs to the anthracycline antibiotic family, one assay method connected with it will be described

here to emphasize the importance of HPLC in these studies. It was of importance to develop a sensitive method to assess the concentration of this drug and its metabolite in plasma and serum. A published method[100] for the determination of aclacinomycin in fermentation broth and drug preparations based on normal phase HPLC was found not to be sensitive enough for this purpose. A new method, based on reversed phase HPLC and fluorescence detection, was developed by Ogasawara et al.[101]. In these experiments standard solutions of aclacinomycin and its metabolites MA 144 S1, MA 144 T1 and MA 144 M1 were prepared in the range of 200 to 1000 ng/ml. Then 100 µl of each of the standards was added to 1 ml portions of plasma and these solutions were diluted with water to different concentrations. Efficiency and reproducibility of the extraction process were based on these samples. Aclacinomycin B, a compound not found among the metabolites, was used for the internal standard and was added to the plasma extracts before the HPLC assay. The extraction process consisted of two successive ethyl acetate extractions at pH 7.0, centrifugation, evaporation of the organic layer and addition of the internal standard. The following chromatographic conditions were used: Instrument, Shimadzu, Model LC-3A; detection, fluorescence detector Model RF-500LC, excitation wavelength 435 nm and emission wavelength 505 nm; column, Bondapak alkyl phenyl; solvent, acetonitrile-0.03 M ammonium formate, pH 5.0, (1:1);

flow rate, 1 ml/min (1000 psi); temperature, 21 ± 1°C; recorder-integrator, Shimadzu Model C-RIA Chromatopak. The system provided baseline separation for all metabolites, straight line calibration curves and a sensitivity limit of 20 ng/ml aclacinomycin in plasma.

A highly sensitive assay was required to estimate the pharmacological behavior of the antitumor antibiotics etoposide and teniposide in humans. Such an assay was developed by Strife et al.[102] under conditions which allowed quantitation of these drugs up to 50 ng/ml serum. Fluorescence detection was used with 215 nm excitation wavelength and 328 nm emission wavelength. The detector was a Schoeffel SF-770 fluorescence detector used with cut-off filters at 300 and 320 nm, 7-54 broad band filter and a narrow band pass interference filter, 8.2 nm wide at half height and centered around 328 nm (Spectrofilm). With the solvent, methanol-water (60:40), good quantitation curves could be obtained despite the failure to achieve baseline separation. The two drugs were used as internal standards for each other.

In an elegant metabolic study, Dye and Rossomando[103] have shown that a salvage mechanism exists for the nucleoside antibiotic formycin A. This antibiotic was phosphorylated to the corresponding mono-, di- and triphosphates with the enzyme adenosine kinase. In this metabolic study HPLC played an important role by providing the possibility of assessing the

quantities of the different phosphorylated products of formycin A. Furthermore it was shown[104] that formycin A triphosphate is a substrate of adenylate cyclase enzyme of rat osteosarcoma. The substrate of this enzymatic reaction was separated from the product, 3',5'-cyclic formycin monophosphate and was quantitated by an HPLC procedure.

Biotransformation by microbiological means is a way to obtain novel derivatives of antibiotics. In such a study[105] the antitumor agent 9-methoxyellipticine was converted to the o-demethylated product and to other metabolites. Also, in another microbial biotransformation study[106] hydroxylation occurred and 8- and 9-hydroxy ellipticines were obtained. The yield of this biological reaction was monitored by an HPLC assay. The assay utilized a Bondapak phenyl column and acetonitrile-0.1% $(NH_4)_2\ CO_3$ solvent.



## LITERATURE

1. A. Aszalos, ed, Antitumor Compounds of Natural Origin: Chemistry and Biochemistry, CRC Press, Boca Raton, FL, 1981.

2. L. C. Craig and J. Sogn, in Methods in Enzymology, Antibiotics, Vol. 43 (J. H. Hash, ed), Academic Press, NY, 1975, p. 320.

3. A. Aszalos and D. Frost, in Methods in Enzymology, Antibiotics, Vol. 43 (J. H. Hash, ed), Academic Press, NY, 1975, p. 172.

4. M. Margosis, J. Chromatogr. Sci. 12, 549, 1974.

5. Cs. Horvath, ed, High Performance Liquid Chromatography, Advances and Perspectives, Vol. 1-2, Academic Press, NY, 1980.

6. L. R. Snyder and J. J. Kirkland, eds, Introduction to Modern Liquid Chromatography (2nd ed.), J. Wiley and Sons, Inc., NY, 1979.

7. M. Verzele, L. De Taeye, J. Van Dyck, G. DeDecker and C. DePauw, J. Chromatogr. 214, 95, 1981.

8. E. Katz, in Antibiotics (D. Gottlieb and P. D. Show, eds), Vol. 2, Springer Verlag, NY, 1967, p. 271.

9. H. Brockman, Pure Appl. Chem. 2, 405, 1961.

10. E. Reich, I. H. Goldberg and M. Rabinowitz, Nature 196, 743, 1962.

11. H. Brochman and H. Grove, Chem. Ber. 87, 1036, 1954.

12. A. W. Johnson and A. B. Mauger, Biochem. J. 73, 535, 1959.

13. W. J. Rzeszotarski and A. B. Mauger, J. Chromatogr. 86, 246, 1973.

14. A. Aszalos and J. Berdy, in Antitumor Compounds of Natural Origin: Chemistry and Biochemistry, CRC Press, Boca Raton, FL, 1981, pp. 77-78.

15. S. Gorog, B. Herenyi and K. Javanovics, J. Chromatogr. 139, 203, 1977.

16. M. Verzele, L. DeTaeye, J. Van Dyck, G. DeDecker and C. DePauw, J. Chromatogr. 214, 95, 1981.

17. J. J. Langone, M. R. D'Onofrio and H. Van Vunekis, Anal. Biochem. 95, 214, 1979.

18. M. C. Castle and J. A. R. Mead, Biochem. Pharm. 27, 37, 1978.

19. N. Ishida, K. Miyazaki, K. Kumagai and M. Rikimaru, J. Antibiot. 18, 68, 1965.

20. T. S. A. Samy, J. Hu, J. Meienhofer, H. Lazarus and R. K. Johnson, J. Natl. Cancer Inst. 58, 1765, 1977.

21. R. Montgomery, V. L. Shepherd and D. D. Vandré, in Antitumor Compounds of Natural Origin: Chemistry and Biochemistry (A. Aszalos, ed) CRC Press, Boca Raton, FL, 1981, p. 79.

22. M. A. Napier, B. Helmquist, D. J. Strydom and I. H. Goldberg, Biochemistry, 20, 5602, 1981.

23. L. S. Kappan, M. A. Napier and I. H. Goldberg, Proc. Natl. Acad. Sci. USA 77, 1970, 1980.

24. H. Umezawa, K. Maeda, T. Takeuchi and Y. Okami, J. Antibiot. Ser. A 19, 200, 1966.

25. I. Kimura, T. Onishi, I. Kunimasa and J. Takano, Cancer (Brussels) 29, 59, 1972.

26. A. Fujii, T. Takita, K. Maeda, and H. Umezawa, J. Antibiot. 26, 396, 1973.

27. A. Aszalos, J. Crawford, P. Vollmer, N. Kantor and T. Alexander, J. Pharm. Sci. 70, 878, 1981.

28. T. T. Sakai, J. Chromatogr. 161, 389, 1978.

29. C. M. Vos, D. Schipper, J. D. M. Herscheid and G. Westere, J. Antibiot. 35, 837, 1982.

30. W. J. Rzeszotarski, W. C. Echelman and K. C. Reba, J. Chromatogr. 124, 88, 1976.

31. G. K. Shin and T. J. Goekl, J. Chromatogr. 181, 127, 1980.

32. T. Hata, Y. Sano, R. Sugawara, A. Matsumae, K. Hakamori, T. Shina and T. Hoshi, J. Antibiot. Ser. A 9, 141, 1956.

33. S. T. Crooke and W. T. Bradner, Cancer Treat. Rev., 3, 121, 1976.

34. S. C. Srivastava and U. Hornemann, J. Chromatogr. 161, 393, 1978.

35. A. Kono, Y. Hara, S. Equchi, M. Tanaka and Y. Matsushima, J. Chromatogr. 164, 404, 1979.

36. M. Tomasz and R. Lipman, Biochemistry 20, 5056, 1981.

37. M. Tomasz and R. Lipman, unpublished results.

38. G. A. van Hazel and J. S. Kovach, 73rd Ann. Meeting Am. Assoc. Cancer Res., April 28 - May 1, 1982, St. Louis, Abstr. No. 489.

39. P. A. Andrews, S. S. Pan and N. R. Bachur, 73rd Ann. Meeting Am. Assoc. Cancer Res., April 28 - May 1, 1982, St. Louis, Abstr. No. 815.

40. A. Aszalos, S. West, T. Alexander and E. Lewis, unpublished results.

41. U. R. Tjaden, J. P. Langenberg, K. Ensing, W. P. VanBennekom, E. A. DeBruijn and A. T. Van Oosterom, J. Chromatogr. 232, 355, 1982.

42. L. E. Broder and S. K. Carter, Ann. Int. Med. 79, 108, 1973.

43. A. B. Adolphe, E. D. Glasofer, W. M. Troetel, A. J. Weiss and R. W. Monthei, J. Clin. Pharmacol. 17, 379, 1977.

44. J. F. Murray, G. R. Gordon and J. H. Peters, Pharmacologist 17, 266, 1975.

45. J. B. Lecaillon, N. Febvre, J. P. Metayer and S. Souppart, J. Chromatogr. 145, 319, 1978.

46. B. Ratti, R. R. Parenti, A. Toselli and L. F. Zerilli, J. Chromatogr. 225, 526, 1981.

47. M. Gidok, S. Tsutsumi and S. Takitani, J. Chromatogr. 223, 379, 1981.

48. V. Vlasakova, J. Benes and K. Zivny, J. Chromatogr. 151, 199, 1978.

49. F. Arcamone, A. G. Franceschi, S. Penco and A. Selva, Tetrahedron Lett. 1969, 1007.

50. A. DiMarco, M. Gaeteni, P. Orezze, B. M. Sarpinato, R. Silverstrini, M. Soldati, J. Dasdia and L. Valentini, Nature 201, 706, 1964.

51. Code of Federal Regulations 21, Food and Drug 436.322, 1980.

52. A. C. Haneke, J. Crawford and A. Aszalos, J. Pharm. Sci. 70, 1112, 1981.

53. E. R. White and J. E. Zarembo, J. Antibiot. 34, 836, 1981.

54. S. D. Averbuch, T. T. Finkelstein, S. E. Fandrich and S. D. Reich, J. Pharm. Sci. 70, 265, 1981.

55. D. E. Nettleton, D. M. Balitz, T. W. Doyle, W. T. Bradner, D. L. Johnson, F. A. O'Herron, R. H. Schreiber, A. B. Coon, J. E. Moseley and R. W. Myllymaki, J. Nat. Prod. 43, 242, 1980.

56. G. K. Poochikian, J. C. Cradock and K. P. Flora, Am. J. Hosp. Pharm. 38, 483, 1981.

57. H. G. Barth and A. Z. Conner, J. Chromatogr. 131, 375, 1977.

58. C. Akpofure, C. A. Riley, J. A. Sinkule and W. E. Evans, J. Chromatogr. 232, 377, 1982.

59. A. Alemanni, U. Breme and A. Vigevani, Process Biochem. 17, 9, 1982.

60. R. Stroshane, M. Guenther, E. C. Piontek and A. Aszalos, ACS National Meeting, Washington, DC, 1979, Abstr. No 44.

61. Code of Federal Regulations 23, Food and Drug 450.22, 1982.

62. R. C. Pandey and M. W. Toussaint, J. Chromatogr. 198, 407, 1980.

63. R. Hulhoven and J. P. Desager, J. Chromatogr. 125, 369, 1976.

64. R. N. Pierce and P. I. Jatlow, J. Chromatogr. 164, 471, 1979.

65. S. Eksborg, H. Ehrsson and I. Andersson, J. Chromatogr. 164, 479, 1979.

66. J. H. Peters and J. F. Murray, Jr., J. Liquid Chromatogr. 2, 45, 1979.

67. S. Shinozawa, Y. Araki and T. Oda, Acta Med. Okayama 35, 395, 1981.

68. J. F. Strauss, R. L. Kitchens, V. W. Petrizi and E. P. Frenkel, J. Chromatogr. 221, 139, 1980.

69. M. J. Sepaniak, E. S. Yeung, J. Chromatogr. 190, 377, 1980.

70. S. Shinozawa and T. Oda, J. Chromatogr. 212, 323, 1981.

71. J. Lankelma, P. G. Penders, J. G. McVie, A. Leyva, W. W. Ten, Bokkel-Huinink, M. M. dePlangua, H. M. Pinedo, Eur. J. Cancer Clin. Oncol. 18, 363, 1982.

72. R. Bocker, J. Chromatogr. 187, 439, 1980.

73. R. Hulhoven and J. P. Desager, Biomedicine 27, 102, 1977.

74. R. Hulhoven, J. P. Desager, G. Sokel and C. Harvengt, Arch. Int. Pharmacodyn. Ther. 226, 344, 1977.

75. J. E. Brown, P. W. Wilkinson and J. R. Brown, J. Chromatogr. 226, 521, 1981.

76. E. Moro, M. G. Jannuzzo, M. Ranghieri, S. Stragnjaick and G. Valzelli, J. Chromatogr. 230, 207, 1982.

77. S. E. Fandrich, J. Chromatogr. 223, 155, 1981.

78. M. Israel, W. T. Pegg, P. M. Wilkinson and M. B. Garnick, J. Liquid Chromatogr. 1, 795, 1978.

79. R. Baurain, A. Zenebergh and A. Tronet, J. Chromatogr. 157, 331, 1978.

80. D. Londos-Gagliardi, R. Baurain, J. Robert and G. Aubel-Sadron, Cancer Chemother. Pharmacol. 9, 45, 1982.

81. H. S. Schwartz and N. B. Parker, Cancer Res. 41, 2343, 1981.

82. S. Shinozawa, T. Fukuda, Y. Araki and T. Oda, Acta Med. Okayama 36, 125, 1982.

83. J. Roboz, A. J. Jacobs, J. F. Holland, G. Deppe and C. J. Cohen, Med. Pediatr. Oncol. 9, 245, 1981.

84. C. M. Camaggi, E. Strocchi, V. Tamassia, A. Mastoni, M. Giovannini, G. Lafelice, N. Canova, D. Marraro, A. Martini and F. Pannuti, Cancer Treat. Rep. 66, 1819, 1982.

85. A. Zenebergh, R. Baurain and A. Trouet, Cancer Chemother. Pharmacol. 8, 243, 1982.

86. S. Eksborg, J. Chromatogr. 149, 225, 1978.

87. R. I. White and K. M. Byrne, J. Antibiot. 35, 529, 1982.

88. R. C. Pandey, M. W. Toussaint, R. M. Stroshane, C. C. Kalita, A. Aszalos, A. L. Garretson, T. T. Wei, K. M. Byrne and R. F. Geoghegen, J. Antibiot. 34, 1389, 1981.

89. A. D. Argoudelis, T. A. Brinkley, T. F. Brodashy, T. A. Buege, H. F. Meyer and S. A. Mizsak, J. Antibiot. 35, 285, 1982.

90. D. M. Balitz, F. A. O'Herron, J. Busk, D. M. Vyas, D. E. Nettleton, R. E. Grulick, W. T. Bradner, T. W. Doyle, E. Arnold and J. Clady, J. Antibiot. 34, 1544, 1981.

91. H. Anke, T. Kemmer and G. Hofle, J. Antibiot. 34, 923, 1981.

92. M. E. Wall, M. C. Wani and H. Taylor, Cancer Treat. Rep. 60, 1011, 1976.

93. K. Tsuji and J. F. Goetz, J. Antibiot. 31, 302, 1978.

94. T. Ogasawa, S. Goto, S. Mori and T. Oki, J. Antibiot. 34, 47, 1981.

95. A. Fujiwara, T. Hoskino, M. Tazoe and M. Fujiwara, J. Antibiot. 34, 608, 1981.

96. N. M. Barfod. J. Chromatogr. 230, 289, 1982.

97. R. T. Wu, T. Okabe, M. Namikoshi, S. Okuda, T. Nishimura and N. Tenaka, J. Antibiot. 35, 279, 1982.

98. N. Naoi, T. Miwa, T. Okazaki, K. Watanabe, T. Takeuchi and H. Umezawa, J. Antibiot. 35, 806, 1982.

99. A. Kono, M. Tanaka, S. Eguchi and Y. Hara, J. Chromatogr. 163, 109, 1979.

100. T. Ogasawa, S. Gato, S. Mori and T. Oki, J. Antibiot. 34, 47, 1981.

101. T. Ogasawara, Y. Mesudo, S. Goto, S. Mori and T. Oki, J. Antibiot. 34, 52, 1981.

102. R. J. Strife, I. Jarife and M. Calvin, J. Chromatogr. 224, 168, 1981.

103. F. J. Dye and E. F. Rossomando, Biosci. Rep. 2, 229, 1982.

104. E. F. Rossomando, J. H. Jabrigen and J. F. Eccleston, Proc. Natl. Acad. Sci. USA, 78, 2278, 1981.

105. M. M. Chien and J. P. Rosazza, J. Nat. Prod. 42, 643, 1979.

106. M. M. Chien and J. P. Rosazza, Appl. Environ. Microbiol. 40, 741, 1980.