Journal of Chromatography, 385 (1987) 99–108
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 988

INTEGRATED BIOLOGICAL–PHYSICOCHEMICAL SYSTEM FOR THE IDENTIFICATION OF ANTITUMOR COMPOUNDS IN FERMENTATION BROTHS

DEREK J. HOOK, CAROLEE F. MORE, JOSEPH J. YACOBUCCI, GEORGE DUBAY and SEAN O'CONNOR

Bristol-Myers Company, Pharmaceutical Research and Development Division and Research Support Unit, 5 Research Parkway, Wallingford, CT 06492 (U.S.A.)

SUMMARY

A rapid, integrated biological–physicochemical system for the identification of six major classes of antitumor compounds in fermentation broths is described. The system relies upon preliminary fractionation of the fermentation broth by liquid–solid extraction, gradient high-performance liquid chromatography with diode-array spectrophotometric detection of the compounds and automated bioassay. The previously stored UV–VIS spectra of the biologically active peaks are used for identification. Confirmation of compound identity is by thermospray liquid chromatography–mass spectrometry. The method has been applid to representatives of the bleomycin, streptonigrin, echinomychin, chromomycin, actinomycin and anthracycline groups.

INTRODUCTION

Fermentation broths are the source of many biologically active species, among them a large number of antitumor compounds. Most methods of identification involve complex time- and labor-intensive procedures to obtain pure or semi-pure samples for the determination of structural type and compound identity.

A number of recent efforts have been directed toward the early identification of compounds in fermentation broths in order to increase the efficiency of screening programs. Two approaches can be taken towards achieving this goal. In the first approach biological test systems with different sensitivity characteristics are used to identify the compounds. In the second approach, a combination of analytical methods is used to separate broth components, followed by a combination of physicochemical and biological identification methods. We have chosen the second approach but have taken advantage of recent advances in instrumental and analytical technology, as well as in biological assay methodology, to improve upon previous methods and to develop an integrated approach to the problem.

In those cases where high-performance liquid chromatography (HPLC) has been used for the identification of antitumor compounds in fermentation broths, the

0021-9673/87/$03.50

procedures have usually been optimized for, and restricted to, the separation and identification of a single family of compounds, *e.g.*, bleomycins[1], anthracyclines[2–4], and actinomycins[5]. The development of diode-array detector technology has only recently allowed the use of on-line UV–VIS spectra as an aid to compound identification during HPLC separation[6,7]. Miller and Neuss[8] used HPLC to separate components of a fermentation broth and collected the effluent on a paper strip, which was dried and used for bioautography. They were able to correlate the location of the biological activity with the retention time of UV peaks, thus achieving identification of the components on the basis of retention time. There are disadvantages to using this method: the effluent is applied to the paper strip through a stream splitter, limiting the amount of material that can be applied, the effluent stream spreads on the paper by diffusion before drying, heat is used for drying, causing possible changes in thermally sensitive compounds, and the biologically active zones are broad, preventing accurate correlation with retention times. In addition, only a conventional, fixed-wavelength detector was used.

Inchaupse *et al.*[9] used a combination of ion-pair HPLC and field-desorption mass spectrometry after effluent collection and tested the biological activity for the early characterization of aminoglycoside antibiotics from fermentation broths. However, specialized ion-pairing reagents had to be used.

Mirabelli *et al.*[10] have described a method by which the growth inhibitory effects of antitumor compounds can be quantitated using tumor cell lines in an *in vitro* assay on microplates. Although they used their method to quantitate the amounts of antitumor agents in fermentation broths, they did this on the crude broth and not on separated components.

We have developed a system by which fermentation broths are first chromatographed to provide a rough index of the lipophilicity of the biologically active material in the broth. An appropriate HPLC system was selected on the basis of this information, and the biologically active fraction was isolated by collecting the eluate in individual wells of a microtiter plate using a commercially available fraction collector. The solvents in the effluent were evaporated in a centrifugal vacuum evaporator and the materials in the individual wells were assayed by *in vitro* microplate assays with either tumor cell lines or by an antimicrobial assay. This procedure provides discrete localization of short segments of the HPLC effluent stream (*ca.* 10 s in span), allowing a more accurate correlation of biological activity with retention time. The diode-array detector permits correlation of the biological activity not only with the UV peaks in the chromatogram but also with the 200–600 nm UV–VIS spectrum of the component.

We have used thermospray liquid chromatography–mass spectrometry (LC–MS) on the material in those wells identified as having biological activity. This method has general applicability to most compound groups, but collecting the effluent has even wider applicability allowing the use of other methods. The information obtained from the combination of these analytical methods has enabled us to identify known antitumor compounds in fermentation broths. This collection of analytical data has enabled us to make exploratory use of advanced data analysis methods to enhance the identification process. We have not greatly increased the concentration of the biologically active materials, which are present in these broths at concentrations typical of those found in routine screening procedures.

EXPERIMENTAL

Materials

Bleomycin was obtained from the Pharmaceutical Research and Development Division (PRDD) of Bristol-Myers (Syracuse, NY, U.S.A.) as Blenoxane; actinomycin from Sigma (St. Louis, MO, U.S.A.); streptonigrin and chromomycin from PRDD of Bristol-Myers (Wallingford, CT, U.S.A.); aclacinomycin and echinomycin from PRDD of Bristol-Myers (Syracuse). Fermentation broths were obtained from the Bristol-Myers Research Institute in Tokyo, Japan. They were produced by fermentation of organisms isolated during the normal course of screening soil organisms for the production of antitumor compounds. A volume of 10 ml of fermentation broth was freeze-dried for shipment, and was reconstituted to the same volume with distilled water, centrifuged, and filtered prior to use.

The tissue culture medium was Gibco No 320-1090 (Gibco, Chagrin Falls, OH, U.S.A.). To 500 ml of this medium were added: 5 ml of 200 m*M* L-glutamine (Sigma) 5 ml penicillin (10000 U/ml), 5 ml streptomycin (10000 μg/ml), 3 ml minimum essential medium (MEM) non-essential amino acids (all 10 m*M*) (Gibco 320-1140) and 55 ml fetal bovine serum. Mueller-Hinton broth was made from powdered material (Difco, Detroit, MI, U.S.A.) according to the manufacturer's directions. B16 and HCT cell lines were obtained from Bristol-Myers (Wallingford).

The C_{18} minicolumns (500 mg in 6-ml columns) were obtained from Analytichem International (Harbor City, CA, U.S.A.). Crystal Violet was obtained from Fisher Scientific (Springfield, NJ, U.S.A.). HPLC-grade water (Burdick & Jackson, Muskegon, MI, U.S.A.) or Milli-Q-purified (Millipore, Bedford, MA, U.S.A.) water was used. HPLC-grade acetonitile and potassium phosphate were obtained from Fisher Scientific.

Chromatography

The fermentation broth samples were chromatographed on 500 mg bonded-phase silica in 6-ml columns (*cf. Materials*). Fractions were collected either manually or by use of a robotic sample preparation system (Zymark, Hopkinton, MA, U.S.A.). The C_{18} column was pre-conditioned by washing with 1 ml acetonitrile, followed by two 1-ml water washes. Then, 4.5 ml of fermentation broth sample was applied to the column and forced through the column using either a vacuum manifold (in the manual method) or air pressure (in the robotic procedure). The elute was collected (fraction 1). The column was then washed with 1 ml water, and the elute was discarded. (The water wash was performed to ensure no carryover of biological activity from the first fraction to subsequent fractions.) The column was successively eluted with 1 ml of 0.015 *M* phosphate buffer (pH 4.5), 1 ml of acetonitrile–phosphate buffer (50:50), and finally with 1 ml of acetonitrile. All three of these fractions were collected (fractions 2–4).

The four fractions were then tested for biological activity using the biological assay methods outlined below, with the difference that serial dilutions of the samples were made before assay and all diluted fractions were assayed for biological activity. The fractions having biological activity were selected for HPLC analysis, as described below.

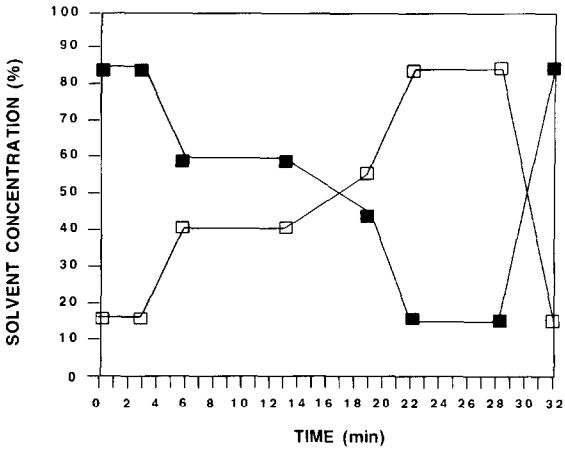

Fig. 1. HPLC gradient: ■ = 0.015 *M* phosphate buffer, pH 3.5, □ = acetonitrile. Conditions as described in Experimental.

HPLC

A Hewlett-Packard (Palo alto, CA, U.S.A.) HP1090 HPLC system was used. This was equipped with a HP1040 diode array spectrophotometric detector and a system controller with a HP85B computer with a hard and floppy disk drive. The column was a Rainin (Woburn, MA, U.S.A.) Microsorb 3-μm C_{18} Short-One.

A gradient system of acetonitrile (pump A) and 0.015 *M* potassium phosphate buffer (pH 3.5) (pump B) was used. The buffer solution was adjusted to the pH specified with phosphoric acid and passes through a 0.45-μm filter before use. The HPLC system was sparged continually with a stream of helium. The gradient program was made up of linear segments and is described in Fig. 1. The flow-rate was 1.2 ml/min, the temperature 23°C. The diode-array detector parameters were: pilot wavelength, 254 nm (bandwidth 4 nm); reference wavelength, 600 nm (bandwidth 25 nm); plot wavelength, 254 nm; timebase, 160 ms; peak width, 0.2; threshold, 4 mAU; bunching, ON; zero offset, 10%; spectral data at apex only from 200 nm to 600 nm (2-nm steps). Spectra were matched visually. Aliquots of 100 μl of the four fractions from bonded-phase chromatography were injected. For the purpose of these experiments duplicate injections were made.

Effluent collection and treatment

The effluent from the outlet of the HPLC equipment was led through a 90-cm segment of 0.3 mm I.D. × 1.5 mm O.D. PTFE tubing (with a dead-volume of 120 μl, resulting in a 0.1-min delay) to a Gilson 212 fraction collector (Gilson Medical Electronics, Middleton, WI, U.S.A.), equipped with the microtiter-plate collection accessory. Two 96-well microtiter plates were placed in positions A and B of the rack assembly, and 10-s fractions were collected. This allowed a complete 32-min HPLC run to be collected in the 192 wells of two microplates. After collection was complete, the microplates were placed in a Savant Speedvac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.) equipped with a microplate rotor, and the the eluate fractions were evaporated to dryness. For the purpose of these experiments duplicate sets of plates were prepared.

Biological assay methods

The material in the wells was reconstituted with 10 μl dimethyl sulfoxide (DMSO) followed by dilution to 200 μl with sterile water. Aliquots were taken for bioassay against either *Bacillus subtilis* ATCC6633 (American Type Culture Collection, Rockville, MD, U.S.A.) or in an *in vitro* cytotoxicity assay against B-16 or HCT tumor cells.

For the antimicrobial bioassay a spore suspension of *Bacillus subtilis,* at the appropriate dilution, was inoculated into sterile Mueller-Hinton broth. Aliquots of 175 μl were dispensed into the wells of a microtiter plate with a Cetus Propette system (Cetus Corporation, Emeryville, CA, U.S.A.). Then, 25 μl of the solutions in the wells of the plate containing the reconstituted HPLC eluate was added to the corresponding wells of the microplate containing the seeded broth. The plates were incubated overnight with shaking in a temperature-controlled cabinet shaker at 35°C (Psycotherm, New Brunswick Scientific, Edison, NJ, U.S.A.). After incubation, the absorbance of the solutions in the wells of the microtiter plate was recorded, using a Titertek Multiskan RC plate reader (Flow Labs., McLean, VA, U.S.A.) with a 540-nm filter. A microplate containing uninoculated Mueller-Hinton broth acted as the blank for the Titertek reader.

For the tumor cell line assay, the B-16 or HCT cells (which are carried in continuous culture) were harvested in the log phase of growth by mild trypsinization, counted, and suspended in growth medium (see *materials*) at a concentration of 20000 cells/ml. Aliquots of 150 μl of this suspension were pipetted into each well of a microtiter plate using the Cetus Propette system and the Cetus MicroTrof tray. The plate was then incubated overnight in a 5% carbon dioxide atmosphere at 37°C in order to produce cells attachment to the bottom of the wells. Then, 25 μl of the solutions from the wells of the plate containing the reconstituted HPLC fractions was added to the corresponding well of the microplate containing the tumor cells.

The assay plates were incubated for 48 h in a 5% carbon dioxide atmosphere at 37°C. During this time any cytotoxic effect of test materials will cause loss of attachment of the cells to the well bottom. Following incubation, the medium was removed by inverting the plate. Cells remaining were fixed at 0°C (with 250 μl methanol per well) for 10 min. After the ethanol was decanted and air-dried (overnight if necessary), 60 μl of dye solution [45 ml stock Crystal Violet in 500 ml of phosphate-buffered saline (MA Bioproducts, Walkersville, MD, U.S.A.), filtered] was added. The dye was removed after 15 min and the wells were twice washed with 250 μl of water by aspiration with a Dynatech Dynawasher II (Dynatech Instruments, Chantilly, VA, U.S.A.). After air-drying, the amount of stain in each well was measured with a Titertek plate reader, a clear plate serving as the blank. If necessary, the wells can be destained by adding 100 μl of a solution of 0.4% acetic acid in water–ethanol (50:50), and after allowing the plates to stand for 10 min, the absorbance is read.

Thermospray LC–MS

A Vestec Thermospray interface (Vestec Corporation, Houston, TX, U.S.A.) connected to a Finnegan MAT 4600TSQ mass spectrometer with a Super Incos Data System (Finnegan Instruments, Sunnyvale, CA, U.S.A.) was used. A solvent stream of 0.1 *M* ammonium acetate buffer (flow-rate, 1.25 ml/min) was pumped into the interface using a Waters 6000A HPLC fitted with a Waters U6K injector (Waters

Assoc., Milford, MA, U.S.A.). Thermospray LC–MS spectra were obtained from standard samples dissolved in acetonitrile and injected directly into the solvent stream. In the case of the fermentation broths, wells of the microplates containing the collected, dried HPLC fractions were selected for examination by thermospray LC–MS if they had shown biological activity in the previous assay. The material in the selected wells was dissolved in 10 μl of the buffer. If necessary, 10 μl of buffer was added to more than one well and the combined material was injected.

The first two quadrupoles of the TSQ were run in the radio frequency only mode, while the third quadropole was scanned from 100 to 1000 daltons in 1.1 s (for compounds with molecular weights > 1000 daltons, the instrument was scanned from 100 to 1500 daltons in 2 s). The source temperature was 248°C and in most cases the interface probe and block temperatures were 235°C and 295°C, respectively. (At times the probe temperature was adjusted if the spectra appeared to be weak or contain excessive fragmentation.) The electron multiplier voltage was *ca.* 2000 v. The instrument was calibrated with gramicidin S as a molecular weight standard and was tuned with a standard sample of aclacinomycin such that the (M + H) ion at 812 was greater than 5% of the base peak at *m/z* 418.

RESULTS AND DISCUSSION

Sample preparation

The robotic sample preparation system is designed to manipulate fermentation broths in test tubes, located in racks placed in the robotic work area. It uses a special hand with a nozzle, connected to solvent lines, and is capable of picking up and manipulating 10-ml pipette tips and 6-ml disposable solid-phase minicolumns. The robot carried out the procedure which generated the four fractions for bioassay. Alternatively, the manipulations were carried out by hand. Sample preparation by a robotic system has the advantages that the samples are treated more reproducibly during the fractionation process and that it allows remote manipulation of potentially hazardous materials.

Following chromatography on the C_{18} minicolumns, the fractions obtained were assayed for *in vitro* activity against the B-16 tumor cell line and against *B.*

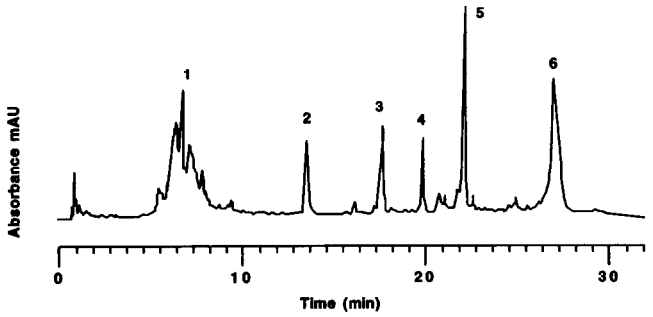

Fig. 2. Chromatogram of six antitumor standards. Peaks (retention time in parentheses): 1 = bleomycin (7.19 min), 2 = streptonigrin (13.59 min), 3 = echinomycin (17.75 min), 4 = chromomycin (19.88 min), 5 = actinomycin D (22.13 min), 6 = aclacinomycin (28.99 min). Conditions as described in Experimental.

subtilis. The results were: no activity in fractins 1 and 2. More than 95% of the biological activity from bleomycin, streptonigrin and echinomycin solutions and broths 1, 2, and 3 were found in fraction 3. Less than 5% of the activity of chromomycin, actinomycin and aclacinomycin solutions and broths 4 and 5 were found in fraction 3. More than 95% of the biological activity from chromomycin, actinomycin and aclacinomycin solutions and broths 4 and 5 were found in fraction 4. Less than 5% of the activity of streptonigrin and chromomycin and broths 2 and 3 were found in fraction 4. These results suggest that bleomycin, streptonigrin and echinomycin were separated from chromomycin, actinomycin and aclacinomycin on the basis of their different lipophilicities and the antitumor compounds could be classified into two groups by this parameter. The fractionation behavior of the biological activities from the different fermentation broths under the same conditions allows a classification of broths 1, 2, and 3 into the group that contains bleomycin, streptonigrin and echinomycin while broths 3 and 4 can be classified into the group that contains chromomycin, actinomycin and aclacinomycin.

HPLC of antitumor standards and fermentation broth fractions

The HPLC procedure outlined under Experimental allows the separation of six major groups of antitumor compounds, as typified by the six standards used. Fig. 2. shows the elution pattern of a mixture of these standards. When the same procedure was applied to the biologically active fractions obtained from preparative chromatography of the fermentation broths, the chromatograms shown in Figs. 3–7 were obtained. After collection of the HPLC effluent in microplates, the various wells were assayed for biological activity, as described under Experimental. These peaks are indicated by asterisks in the chromatograms shown.

Fig. 8a–e shows the absorption spectra of the peaks corresponding to the antitumor standards (*cf.* Fig. 2). The absorption spectra of the corresponding biologically active peaks in the chromatograms of the fermentation broth fractions are shown in Fig. 8f–j. The spectra and retention times (given in the caption) clearly match the active components with those of the corresponding standard antitumor compounds. Aclacinomycin (not shown) was not present in the broths, but it may

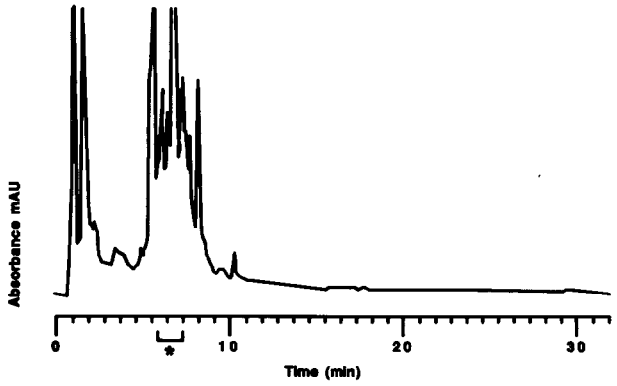

Fig. 3. Chromatogram of fraction 3 from fermentation broth 1. Biological activity indicated by asterisk. Retention time for this peak: 7.29 min. Conditions as described in Experimental.

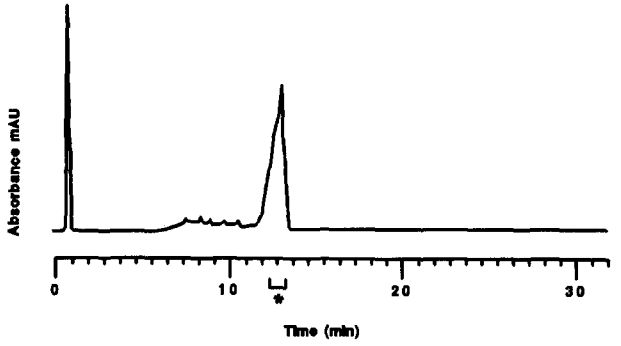

Fig. 4. Chromatogram of fraction 3 from fermentation broth 2. Biological activity indicated by asterisk. Retention time for this peak: 13.04 min. Conditions as described in Experimental.

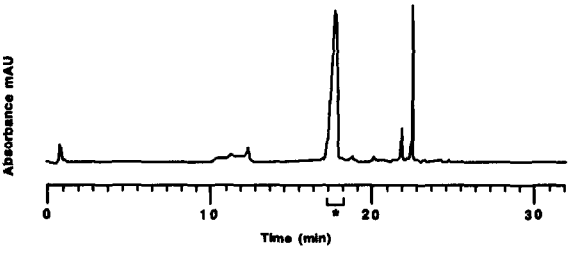

Fig. 5. Chromatogram of fraction 3 from fermentation broth 3. Biological activity indicated by asterisk. Retention time for this peak: 17.78 min. Conditions as described in Experimental.

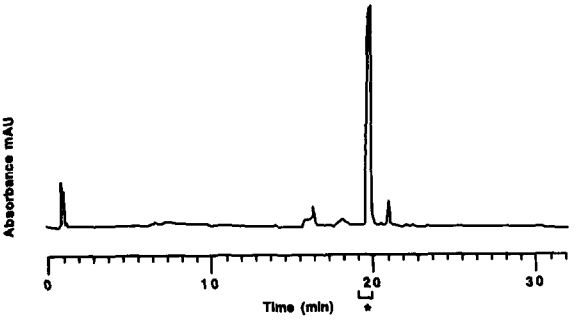

Fig. 6. Chromatogram of fraction 4 from fermentation broth 4. Biological activity indicated by asterisk. Retention time for this peak: 19.82 min. Conditions as described in Experimental.

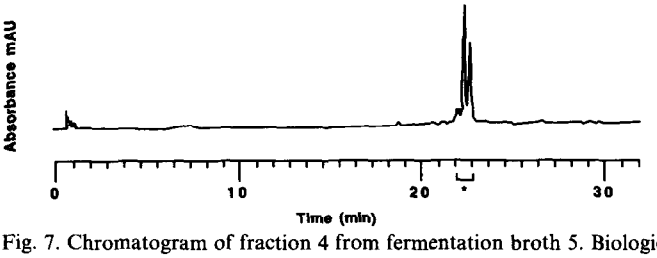

Fig. 7. Chromatogram of fraction 4 from fermentation broth 5. Biological activity indicated by asterisk. Retention time for this peak: 22.48 min. Conditions as described in Experimental.

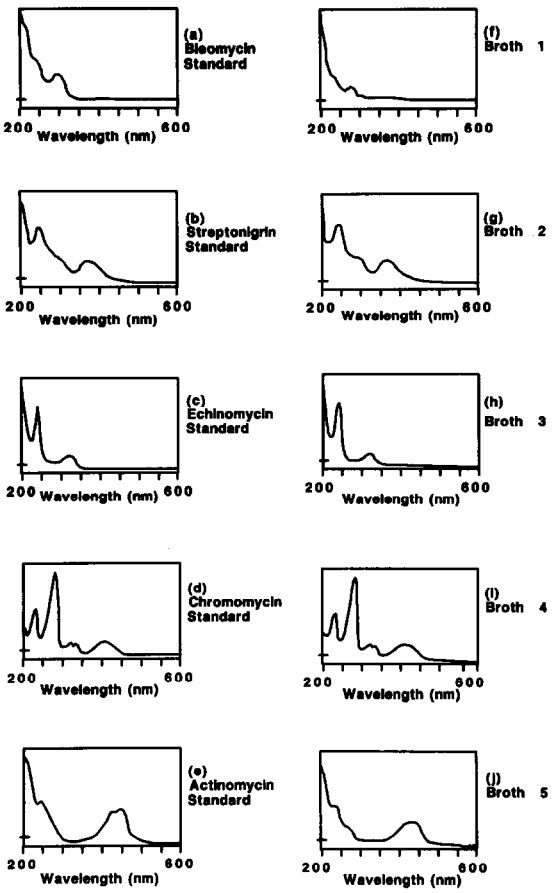

Fig. 8. UV–VIS spectra of peaks of standard antitumor compounds from Fig. 2 and bioactive peaks from Figs. 3–7. Diagrams a–e correspond to bleomycin, streptonigrin, echinomycin, chromomycin and actinomycin D, respectively (retentions times as indicated in the legend of Fig. 2). Diagrams f–j correspond to the bioactive peaks from broths 1–5, respectively (retention times as indicated in the legends of Figs. 3–7, respectively. Matching of spectra were done visually. Other conditions as described in Experimental. Retention times: (a) 7.19, (b), 13.59, (c) 17.75, (d) 19.88, (e) 22.13, (f) 7.29, (g) 13.04, (h) 17.78, (i) 19.82, (j) 22.48 min.

be concluded that the active material in broth 1 belongs to the bleomycin family, broth 2 to the streptonigrin family, broth 3 to the echinomycin family, broth 4 to the chromomycin family, and broth 5 to the actinomycin family.

Bioassay of HPLC effluent

Results obtained from the bioassay of the fractions from the chromatogram in Fig. 2 showed a typical cytotoxic pattern against HCT cells in the *in vitro* assay. The readings from the microplate reader were transformed by a computer program into a graphical representation of bioactivity. A similar graphical representation was obtained when *B. subtilis* was used in the assay procedure.

Thermospray LC–MS

In order to confirm the classification of the biologically active components, thermospray LC–MS was applied on material from the microplates containing the HPLC effluent from the bioactive fractions broths 3 and 5. The mass spectral data supported the structure assignments made on the basis of the UV–VIS spectra in Fig. 8h and j and the retention time data.

Our results indicate that thermospray LC–MS is widely applicable to the identification of small quantities of material, typical of those found in fermentation broths, and that little sample preparation and work-up is necessary. Only small amounts of standards were necessary to obtain good spectra by this technique, in the range of 10–20 μg per injection.

CONCLUSIONS

This paper demonstrates that a synthesis of the techniques of solid–liquid extraction, gradient HPLC with a diode-array detector, collection and bioassay of the HPLC effluent and thermospray LC–MS can provide an enhanced ability to identify antitumor compounds from fermentation broths than any of the techniques in isolation.

The methods described are applicable to the identification of the many types of compounds found to be active in the wide variety of screens typical of a modern fermentation screening program.

ACKNOWLEDGEMENTS

The thermospray LC–MS experiments were done under contract at Oneida Research Services, Utica, NY, U.S.A. We thank our colleagues at the Bristol-Meyers Research Institute (Tokyo, Japan) for their interest and support and for supplying the broth samples used in this study.

REFERENCES

1 A. Alemanni and M. Riedmann, *Chromatographia*, 12 (1979) 396.
3 M. A. Van Lancker, H. J. C. F. Nelis and A. P. De Leenheer, *J. Chromatogr.*, 254 (1983) 45.
4 S. Eksborg, *J. Chromatogr.*, 149 (1978) 225.
5 W. J. Rzeszotarski and A. B. Mauger, *J. Chromatogr.*, 86 (1973) 246.
6 F. Overzet, A. Rurak, H. Van der Voet, B. F. H. Drenth, R. T. Ghijsen and R. A. De Zeeuw, *J. Chromatogr.*, 267 (1983) 329.
7 K. Zech, R. Huber and H. Elgass, *J. Chromatogr.*, 282 (1983) 161.
8 R. D. Miller and N. Neuss, *J. Antibiot.*, 31 (1978) 1132.
9 G. Inchaupse, C. Dehayes and D. Samain, *J. Antibiot.*, 38 (1985) 1526.
10 C. K. Mirabelli, H. Bartus, J. O. L. Bartus, R. Johnson, S. M. Mong, C. P. Sung and S. T. Crooke, *J. Antibiot.*, 38 (1985) 758.