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Note

Confirmatory identification of Susceptibility Card antibiotics by ion-pair and reversed-phase high-performance liquid chromatography

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The clinical efficiency of antibiotic therapy can be dramatically enhanced by optimization of the antibotic selection process based on the use of rapid antibiotic susceptibility tests. Susceptibility Cards, developed and produced by Vitek, are disposable antibiotic-susceptibility test cards which contain eight different antibiotics and a nutrient broth positioned in individual wells of a plastic card. The use of these cards in conjunction with Vitek's Automicrobic System (AMS™) permits automated determination of the susceptibility of pathogenic microorganisms to different antibiotics. Because of the reliance which will be placed upon information elicited from their use, confirmatory evidence of the labeled identity of the antibiotics in their designated cavity positions is required by the U.S. Food and Drug Administration as part of the production quality control for each batch of cards produced.

The individual wells of the card contain a proprietary nutrient-broth mixture plus submicrogram quantities of different antibiotics and chemoterapeutic agents; Fig. 1 is an *Escherichia coli* Susceptibility Card.

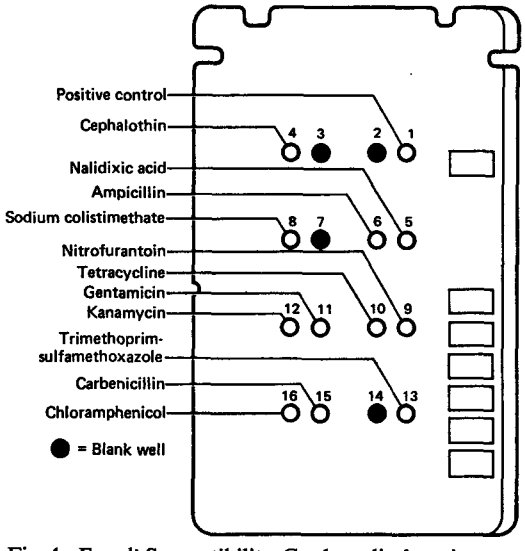


Fig. 1. *E. coli* Susceptibility Card media locations and well numbers,

The conventional approaches to antibiotic identification include three stages: (1) isolation of the individual antibiotic from other components of the mixture (nutrient broth), (2) detection at a submicrogram level, and (3) registration of characteristic molecular properties.

Thin-layer chromatography (TLC) separation of the antibiotics from the nutrient broth followed by chemical[1] or microbiological detection and determination of the antibiotic R_F values are the basis for the currently used procedures for confirmatory identification. These procedures are time consuming and therefore cannot be considered cost effective. High-performance liquid chromatography (HPLC) can facilitate faster separation of complex mixtures than provided by TLC and therefore is a potential cost-effective method of identifying the Susceptibility Card antibiotics. This paper describes the development of reversed-phase and ion-pair HPLC procedures for identification of five susceptibility card antibiotics.

EXPERIMENTAL

Approach

The sequence of experiments performed for the development of rapid HPLC procedures to identify Susceptibility Card antibiotics consists of: (1) Determining retention behavior of individual antibiotics on a bonded-phase column with different solvent systems. (2) Determining the chromatographic profile of the nutrient broth under conditions that produce favorable retention for given antibiotics on the given column. (3) Optimizing broth–antibiotic separation. For those antibiotics that did not exhibit favorable retention with the first bonded-phase column (μBondapak C_{18}), the same sequence of experiments was repeated with a second bonded-phase LC column (μBondapak NH_2). Finally, the antibiotics that produced unfavorable retention behavior with the first two LC columns were submitted to the above sequence with a third, different LC column (μBondapak CN).

HPLC instrument details

All experiments were performed with a liquid chromatograph (Waters Assoc. Model ALC 202) which is equipped with two high-pressure pumps for gradient elution, an injector (Waters U6K), a fixed-wavelength UV detector operating a 254 nm and a variable-wavelength UV detector (Schoeffel Model 770).

Bonded-phase LC columns

Since most of the antibiotics of interest are not soluble in organic solvents and are soluble in water, bonded-phase LC columns which can be used with aqueous solvents were selected as the most suitable[2]. Currently, three chemically different types of commercially available bonded-phase columns are most widely used: (1) bonded-phase LC columns packed with 10-μm porous silica particles to which a layer of C_{18} hydrocarbon has been chemically bonded by reacting octadecyltrichlorosilane with the porous silica (μBondapak C_{18}), (2) bonded-phase LC columns packed with 10-μm porous silica to which a layer of propylamine has been chemically bonded by reacting aminopropylsilane with the porous silica (μBondapak NH_2) and (3) bonded-phase LC columns packed with 10-μm porous silica to which a layer of propionitrile has been chemically bonded (μBondapak CN).

Calibration solutions

Determination of antibiotic retention behavior was performed by injection of 10 μl of different calibration solutions. These solutions were prepared by weighing *ca.* 10 mg of a particular antibiotic in a vial and adding 5% methanol in distilled water for an antibiotic solution concentration of 0.5 μg/μl. The resulting antibiotic solutions were stored in a freezer at $-5°$ to $-10°$.

Sampling techniques

Pure broth or broth-plus-antibiotic samples from the Suscepibility Card are analyzed by the following procedure. Distilled water (25 μl) is added to the well to dissolve its content. Approximately 15 μl of the well solution are withdrawn and redeposited to mix the well content. This procedure is repeated three to four times. Then 20 μl of the well content are withdrawn and injected into the liquid chromatograph through the injector.

Mobile phase solvents

The individual HPLC separations are performed with the specific combinations of solvents given in Table I. All solvents were filtered through a 0.45-μm filter using a solvent filtering kit (Waters Assoc.) and were continuously stirred with a magnetic stirrer to eliminate dissolved gas.

TABLE I

CHROMATOGRAPHIC CONDITIONS FOR HPLC IDENTIFICATION OF ANTIBIOTICS

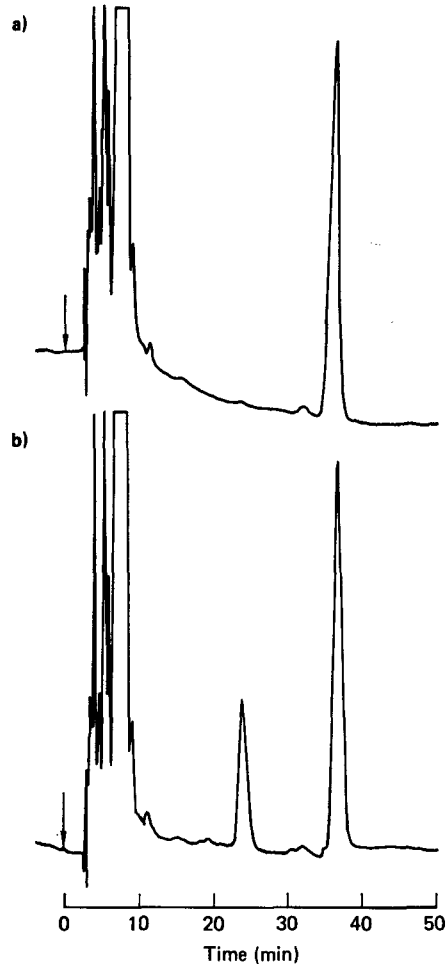
Antibiotic	*LC column type*	*Solvent*	*Flow-rate (ml/min)*	*Antibiotic retention time (min)*	*Remarks*
Ampicillin	μBondapak C_{18} (Waters Assoc.)	300 ml methanol, 700 ml water, 20 ml PIC Reagent A (Waters Assoc.)	1	23.6	ion-pair reversed-phase chromatography permits retention of ionic molecules
Chlor-amphenicol	μBondapak C_{18} (Waters Assoc.)	300 ml methanol, 700 ml water, 20 ml PIC Reagent A (Waters Assoc.)	1	20.1	ion-pair reversed-phase chromatography permits retention of ionic molecules
Carbenzillin	μBondapak NH_2 (Waters Assoc.)	89 ml acetic acid, 40 ml methanol, 500 ml acetonitrile, 375 ml water	1	37.4	separation achieved possibly in the ion-exchange mode
Cephalothin	μBondapak NH_2 (Waters Assoc.)	89 ml acetic acid, 40 ml methanol, 500 ml acetonitrile, 375 ml water	0.6	17.7	separation achieved possibly in the ion-exchange mode
Tetracycline	μBondapak CN (Waters Assoc.)	150 ml methanol, 150 ml THF, 700 ml water, 7 ml acetic acid, 0.21 g EDTA	1	16.9	reversed-phase chromatography with EDTA added to eliminate tetracycline complexation with active sites of the metal surface

Reagents for performing ion-pair HPLC separations are commercially available from Waters Assoc. and are conveniently prepacked for easy preparation of ion-pair solvents.

RESULTS AND DISCUSSION

The development of HPLC procedures to identify Susceptibility Card antibiotics consisted of determining a suitable combination of chromatographic conditions to produce the broth–antibiotic separation.

HPLC conditions that produce broth–antibiotic separation under long retention times (more than 90 min) for the antibiotic cannot be considered acceptable because they would result in chromatographic-peak broadening and a considerable loss of sensitivity. HPLC conditions that produce broth–antibiotic separation under



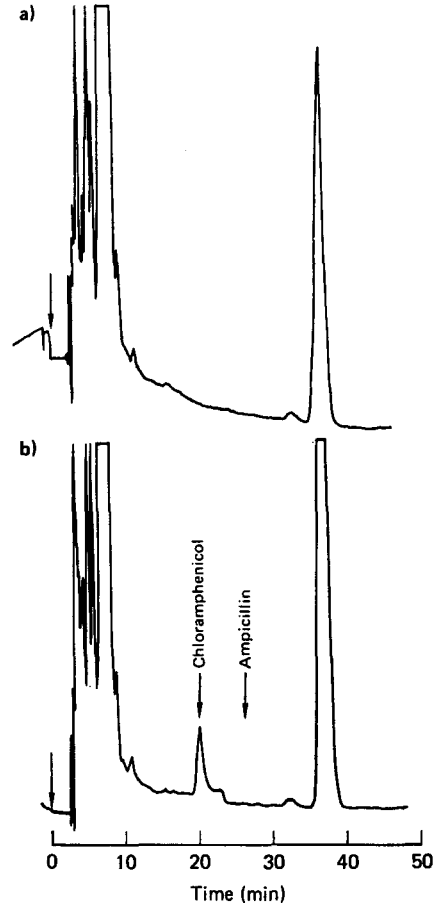


Fig. 2. Chromatographic profiles of broth (a), and broth plus ampicillin (b).

Fig. 3. Chromatographic profiles of broth (a), and broth plus chloramphenicol (b).

short retention times for the antibiotic also cannot be considered acceptable because the retention time will not be a reliable source of characteristic retention information required for the confirmatory identification.

From the exploratory phases of the program, it was concluded that the rapid elution or nonretention of the antibiotics under most chromatographic conditions represents a major difficulty in the development of HPLC procedures for the confirmatory identification of antibiotics. Attention was therefore focused on the new and evolving HPLC technique of ion-pair chromatography which facilitates increased retention of highly polar and ionic compounds under reversed-phase conditions[3,4]. The application of ion-pair, reversed-phase HPLC led to successful development of a HPLC procedure for confirmatory identification of ampicillin and chloramphenicol with a μBondapak C_{18} column. Figs. 2 and 3 show the chromatographic profiles of the nutrient broth, and the broth plus antibiotic for ampicillin and chloramphenicol. Both antibiotics are well separated from all the constituents of the nutrient broth,

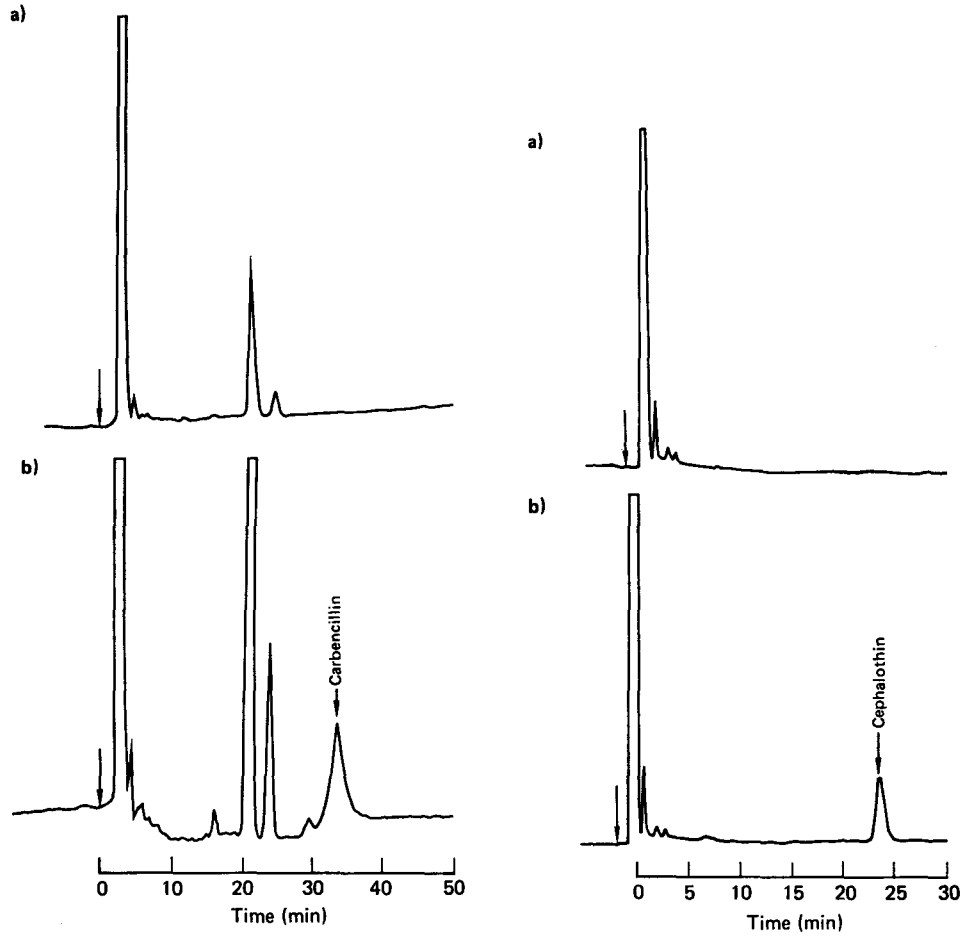


Fig. 4. Chromatographic profiles of broth (a), and broth plus carbencillin (b).

Fig. 5. Chromatographic profiles of broth (a), and broth plus cephalothin (b).

and the difference in their retention times is sufficiently large (3.5 min) to permit conclusive confirmation of their identities.

Separation of the broth–antibiotic mixture for cephalothin and carbencillin is based on a variation of the HPLC conditions developed by Miller and Neuss[5] for separation of celalosporin antibiotics. Cephalothin and carbencillin were separated from the nutrient broth using a μBondapak NH_2 column under conditions given in Table I. Figs. 4 and 5 show the chromatographic profiles of the nutrient broth, and the broth plus antibiotic for cephalothin and carbencillin.

The experiments conducted under "column 3" sequence (μBondapak CN) led to the successful development of an HPLC procedure for intermediate retention of tetracycline under the chromatographic conditions summarized in Table I. The chromatographic profiles of the broth, and the broth plus tetracycline are shown in Fig. 6.

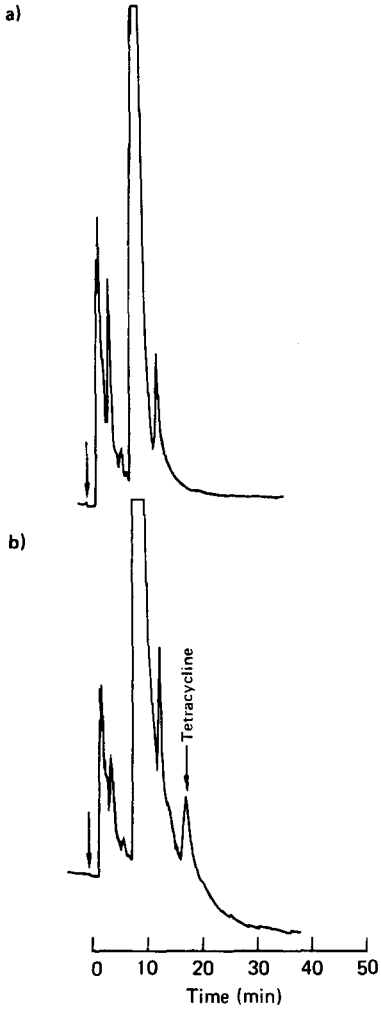


Fig. 6. Chromatographic profiles of broth (a), and broth plus tetracycline (b).

The results described in this note indicate that HPLC facilitates separation of the broth–antibiotic mixture and permits rapid confirmatory identification of susceptibility card antibiotics.

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