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AN AUTOMATED LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF TETRACYCLINE ANTIBIOTICS

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SUMMARY

A liquid chromatographic method for determining the tetracycline antibiotics was automated. The technique employed the use of a valve arrangement through which twenty to thirty samples in solution were sequentially applied to a single column for chromatography. Each chromatogram consisted of a continuous flow separation of components followed by a spectral determination of the column eluate at a rate of twelve samples per hour. The method was applicable to crystalline tetracycline antibiotics and their various pharmaceutical dosage forms.

INTRODUCTION

The most commonly used and official method for determining tetracycline potency is the microbiological[1–3] assay procedure. In addition to the microbiological procedure, numerous other methods including non-aqueous titrimetry[4], UV spectroscopy[5–9] and fluorometry[10] have exhibited some usefulness. However, these methods are subject to limitations. Tetracyclines are known to be contaminated with decomposition products and other closely related compounds, often differing by as little as one functional group. Because these procedures are not specific and they cannot distinguish between similar tetracyclines, decomposition products and other closely related compounds, it requires that some type of quantitative separation technique must be used prior to the determination of the intact tetracycline. This has stimulated attempts to develop the more accurate and specific chromatographic[11–19] methods of analysis.

Interest in this laboratory for the determination of tetracycline purity made necessary the development of a column chromatographic[12] (CC) technique. The column method proved to be specific and accurate for the determination of tetracyclines but had the limitation of a single analysis per column. Since CC was time-consuming and required controlled conditions, and the need for assaying large numbers of samples, an investigation of the feasibility of automating a column procedure was initiated.

This column procedure[12] was successfully converted to an automated system which provided sequential sample application, separation and spectrophotometric determination of eluates in a continuous fashion using a single column. Although the

method is not expected to be a substitute for one providing separate analysis for decomposition products and closely related compounds, the features of ease of column preparation, rapidity of analysis and repetitive use of the same column makes it a technique highly desirable for the determination of tetracycline purity. The advantages of specificity, accuracy, speed and precision normally inherent to automated chromatography were realized.

MATERIAL AND METHODS

Apparatus

The apparatus used in the automated CC includes the following components: (a) Cheminert Metering Pump Model CMP-3, automatic-sample-injection valve Model SVA-8031, three-way valve Model CAV-3031, Cheminert Column Model LC-½-13, polytetrafluoroethylene tubing 0.031 in. and 0.063 in. bore, pneumatic actuators Model PA-875, solenoid air valves and flanging tool (Chromatronix Inc., Berkeley, Calif.); (b) multiple-program cycling timer Model 540 (Conrac Corp., Old Saybrook, Conn.); (c) Sampler II, proportioning pump, assorted tubing and glass fittings (Technicon Corp., Tarrytown, N.Y.); (d) spectrophotometer DBG, equipped with 4 mm rectangular flow cell (Beckman Instruments, Inc., Fullerton, Calif.); (e) recorder, modified with a linear retransmitting slidewire (Bristol Co., Waterbury, Conn.); (f) digital integrator Model 3370A (Hewlett-Packard, Avondale, Pa.)

Reagents

EDTA, pH 6.0. Dissolve 37.2 g of ethylenediaminetetraacetic acid disodium salt in *ca.* 800 ml of distilled water. Adjust the pH to 6.0 with ammonium hydroxide and dilute to 1 l with distilled water.

Polyethylene glycol 400 (PEG 400), 20 % in glycerine. To 80 ml of glycerine, add sufficient PEG 400 to make 100 ml. Mix well.

Buffer. To 95 ml of EDTA pH 6.0, add sufficient 20 % PEG 400 in glycerine to make 100 ml. Shake well.

Calcium lactate–morpholine solution (CLM). Dissolve 0.30 g of calcium lactate in 450 ml of methanol. Add 4 ml of morpholine, swirl and dilute to 500 ml with methanol.

Working reference standard. Prepare a solution containing 0.6 mg/ml of reference standard by dissolving 60 mg of powder in 10 ml of methanol and diluting to 100 ml with chloroform.

Methanol, chloroform, benzene and morpholine, reagent grade.

Acid-washed diatomaceous earth (marketed as Celite® 545 by Johns-Manville Co.). Preparation has been described previously[12].

Preparation of column support

Mix 100 g of acid-washed diatomaceous earth with 30 ml of buffer, in a plastic bag or in a glass jar. Roll or mix until the support is completely homogenous.

Column preparation

Place inlet assembly into the LC-½-13 column and secure the collar to the column. Weight 8.0 ± 0.1 g of column support and add to the column in four 2.0 g

portions. After each addition settle the support by lightly tapping the inlet collar of the column on a padded bench top. Then firmly pack each portion tightly with a tamping rod. The column height should be 12.4 cm.

Preparation of developing solvent

Mix vigorously appropriate amounts of solvents and buffer (see Table I) in a separatory funnel. Allow the two phases to separate and equilibrate for 1 h. Use the organic phase as the developing solvent.

TABLE I

AUTOMATED COLUMN SYSTEMS USED TO DETERMINE TETRACYCLINE ANTIBIOTICS

Antibiotic	*Column packing*	*Developing solvent*	*Maximum absorption-wavelength (nm)*	*Flow rate (ml/min)*
Chlortetracycline HCl	8.0 g column support; 12.4 cm length	Chloroform–benzene–buffer (850:150:100)	393	5
Tetracycline HCl	8.0 g column support; 12.4 cm length	Chloroform–buffer (1000:100)	385	5
Demethylchlortetracycline HCl	8.0 g column support; 12.4 cm length	Chloroform–benzene–buffer (900:100:100)	390	5

Sample preparation

Take an accurate amount of sample equivalent to 60 mg of tetracycline antibiotic. Transfer to a 100-ml volumetric flask and add 10 ml of methanol. Swirl the contents until solution is complete and dilute to volume with chloroform. If filtration is necessary use Whatman No. 541.

Automated column procedure

The automated column systems for the determination of tetracyclines are listed in Table I. The modular assembly and flow diagram representing the arrangement of the automated equipment for the analytical procedures are illustrated in Fig. 1. Working reference standards of tetracyclines were placed on the sampler tray followed by samples. Additional reference standards were interspaced among test samples to maintain calibration of the system. The sample probe of the sampler was aligned so that satisfactory aspiration from aluminum-foil-covered sample cups and rinsing compartment occurred. Before operational startup the metering pump was set at a rate of 5 ml/min, remote timer at twelve samples per h and automatic-sample-injection valve in position A, fill sample loop (see Fig. 5). For operational startup, the column was purged with developing solvent and a steady base line was established with the following modules in operation: metering pump, proportioning pump, spectrophotometer and recorder. After the column was purged (no more air bubbles evident at the AO connector, approximately 5–10 min) and a steady base line had been obtained, the slider of the sample injection valve was activated to position B, inject sample (see Fig. 5). Then the integrator and the remote timer were started.

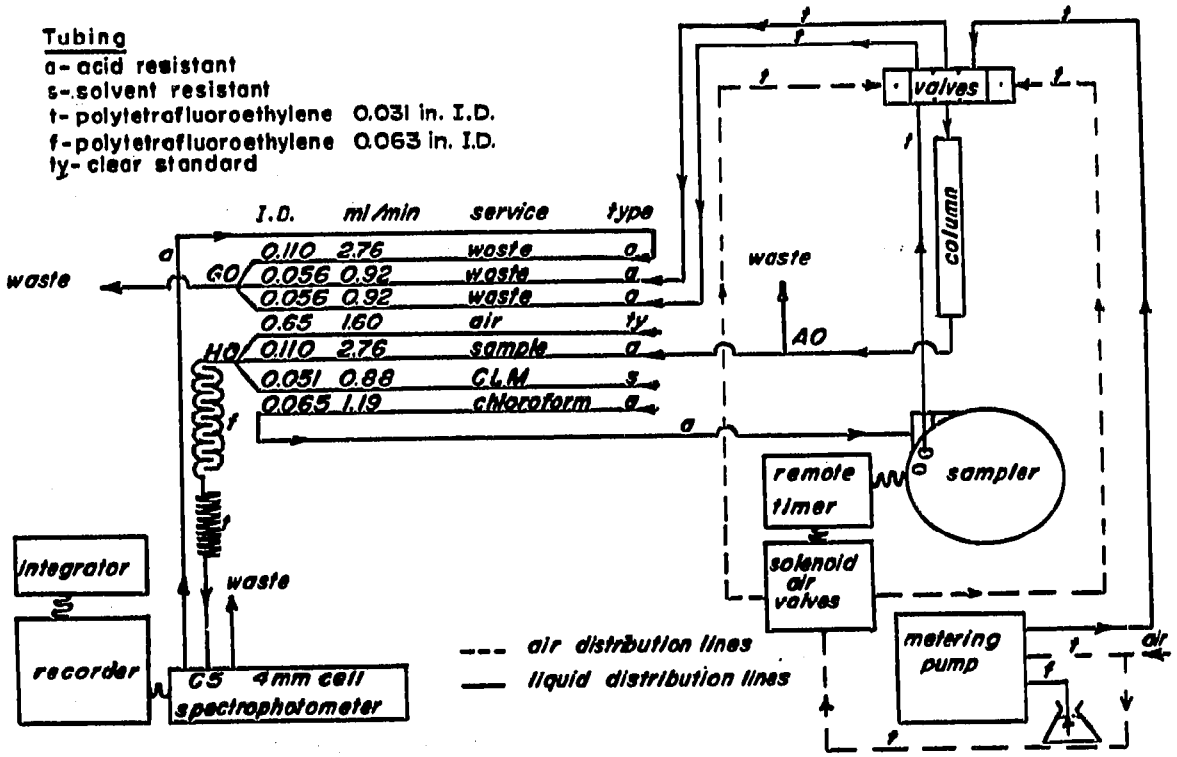

Fig. 1. Modular assembly and flow diagram representing the equipment arrangement for the automated chromatography of tetracyclines.

When the remote timer was actuated the valve slider moved to position A. Simultaneously, the sample probe pierced the aluminum foil and dipped into the sample cup. Sample was drawn from the cup into the sample loop while the developing solvent flowed to the column via the bypass loop. After 1 min 40 sec the remote timer activated the slider back to position B. The sample now contained in the sample loop was forced onto the column by the developing solvent. At the same time the sample probe returned to the rinsing compartment and the sample tray was indexed for the next sample. The column effluent from the chromatogram passed to the AO connector from which a portion of eluate was continually drawn into the manifold system, segmented with air, and joined by a stream of CLM solution. The reaction mixture passed through a polytetrafluoroethylene mixing coil and was monitored by the spectrophotometer. The output of the spectrophotometer was fed into a strip chart recorder and then to the integrator. For the measurement of the peak areas the Hewlett-Packard integrator was used with the following adjustments: Noise suppression, maximal; slope sensitivity, up 0.1 mV/min, down 0.1 mV/min; peak level, 1000 mV; shoulder control, front on, rear, 1000 mV; base line reset delay, 0. The chromatogram was visually displayed on the strip chart but calculations were made by comparing integrated data of standards with samples.

RESULTS AND DISCUSSION

Earlier work[11,12] on the separation and determination of tetracyclines by CC suggested the development of this automated liquid chromatographic technique. In developing the automated column, various modifications were made to adapt the

basic CC system. The resolution and speed of repetitive chromatograms was increased by optimizing the parameters of support, column length, solvents, flow rates, sample preparation and sample size. After several attempts the chromatographic systems shown in the recommended method were developed. It was ascertained that 8.0 g of acid-washed diatomaceous earth, impregnated with buffer was sufficient to pack a column 12.4 cm in length. A constant flow rate of developing solvent, 5 ml/min, was passed through a single column to obtain 20–30 chromatograms.

The determination of each chromatogram was accomplished by monitoring the column eluate with CLM through a spectrophotometric flow cell of a spectrophotometer. The resultant spectrophotometric response was recorded on a recorder and then retransmitted to a digital integrator for quantification. The eluted tetracycline, when mixed with CLM reagent, formed a tetracycline–calcium complex. The absorption spectrum of this tetracycline complex was determined on a Cary II recording spectrophotometer. Fig. 2 shows the absorption spectra of the tetracycline–calcium complexes which had a hypochromic as well as a hyperchromic effect with maximum absorption obtained immediately. The wavelengths where maximum absorption occurred when CLM reagent was introduced into the tetracycline chromaphoric system were as follows: demethylchlortetracycline, 390 nm; tetracycline, 385 nm; chlortetracycline, 393 nm. This absorptiometric system remained constant for a much greater length of time than the system previously described[12].

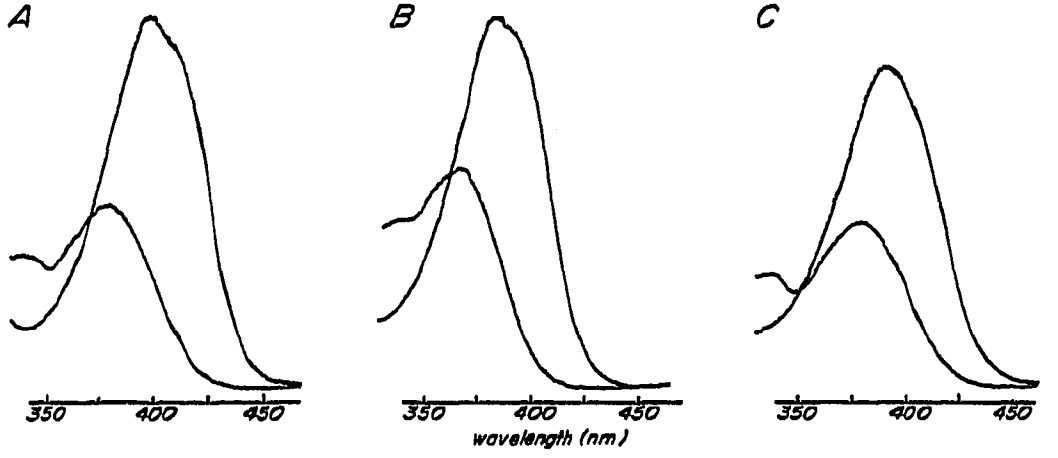

Fig. 2. Absorption spectra of tetracyclines and tetracycline–calcium complexes. (A) Demethylchlortetracycline HCl; (B) tetracycline HCl; (C) chlortetracycline HCl.

Standard concentration curves and samples of tetracycline were prepared under the conditions of the automated column procedure described. Fig. 3 shows the response of each chromatogram obtained from three concentration levels of each reference standard and sample of tetracyclines. The chromatographic responses illustrated that the separation of tetracyclines from related compounds was ideal. The integrated area of standards as microvolt seconds, and concentrations represented in Fig. 3 can be found in Table II. When the measured peak areas of the standards were plotted, a linear relationship existed between microvolt seconds and concentration.

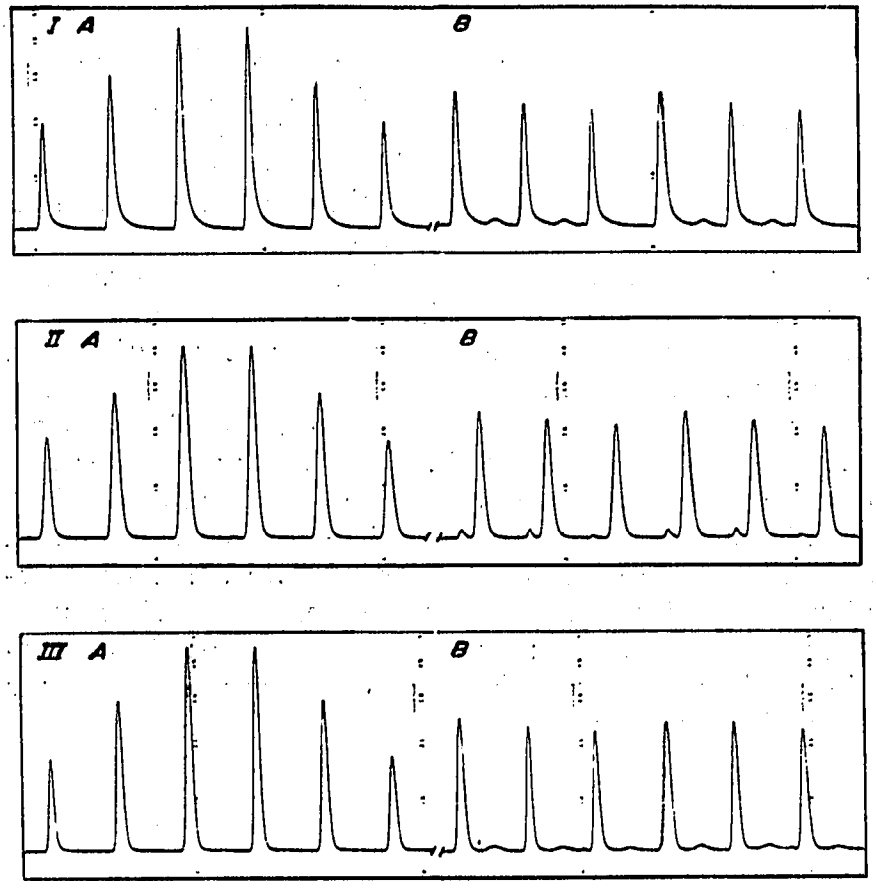

Fig. 3. Recordings of reference standard calibration curves (three concentration levels) and sample curves of tetracyclines (I) Chlortetracycline HCl; A = standard, B = sample. (II) Tetracycline HCl; A = standard, B = sample. (III) Demethylchlortetracycline HCl; A = standard, B = sample.

TABLE II

LINEARITY STUDY OF DATA FROM AUTOMATED CC OF TETRACYCLINES

Units compared	*Linearity of data*								
	Chlortetracycline HCl			*Tetracycline HCl*			*Demethylchlortetracycline HCl*		
Concentration (mg/100 ml)	39.58	58.82	78.95	39.40	59.14	78.87	29.66	49.59	69.7(
Mean microvolt sec ($\times 10^4$)	36.33	54.08	72.47	42.21	63.03	83.05	30.99	51.01	72.0:

A statistical study for precision and accuracy was made. Twenty-four replicates of each tetracycline standard were determined for precision using μV sec/mg as the unit of measurement. Tetracycline standards (amount in mg known from theoretical considerations) were assayed in quadruplicate for the purpose of accuracy. The data from precision and accuracy studies were statistically analyzed and are summarized in Tables III and IV. As may be seen from Table III, the overall precision had a coefficient of variation of 1.01 with a standard error estimated to be 0.144. Table IV

lists the average of the four determinations, the standard error, the deviation from theory and the 95 % confidence limits. 95 % confidence limits are within ± 2 % of theory except chlortetracycline HCl, sample No. 3.

TABLE III

PRECISION STUDY OF DATA FROM AUTOMATED CC OF TETRACYLINES

Tetracycline antibiotic	*No. of observation*	*Mean uV sec/mg*	*Standard error*	*Coefficient of variation*
Tetracycline HCl	24	10623.4	19.92	0.92
Demethylchlortetracycline HCl	24	10354.3	22.28	1.05
Chlortetracycline HCl	24	9208.4	20.15	1.07

TABLE IV

ACCURACY STUDY OF DATA FROM AUTOMATED CC OF TETRACYCLINES

Tetracycline antibiotic	*Sample No.*	*Theoretical amount (mg)*	*Determinations (mg)*			*Deviation from theory*		
			No.	*Mean*	*Standard error*	*Estimate (%)*	*95% Conf. limits*	
							Lower (%)	*Upper (%)*
Tetracycline HCl	1	53.20	4	53.16	0.16	−0.08	−0.68	0.52
	2	49.50	4	49.53	0.12	0.06	−0.43	0.55
	3	47.09	4	46.83	0.19	−0.55	−1.35	0.26
Demethylchlortetracycline HCl	1	44.54	4	44.64	0.18	0.22	−0.59	1.03
	2	42.37	4	42.43	0.14	0.14	−0.54	0.81
	3	39.91	4	39.78	0.26	−0.31	−1.62	1.00
Chlortetracycline HCl	1	53.12	4	52.95	0.17	−0.27	−0.93	0.38
	2	47.81	4	48.21	0.25	0.85	−0.22	1.91
	3	44.15	4	44.62	0.33	1.06	−0.45	2.57

Samples of tetracyclines were dissolved in methanol and then diluted with chloroform. Although this sample preparation was both simple and efficient, several mechanical and chromatographic problems arose due to the use of chloroform. 1 in. light-weight aluminum foil squares which were held in place by a suitable plastic ring were placed over 3.0-ml polyethylene cups. Placing the aluminum foil over the cups of chloroform, eliminated the problem of evaporation. The sample probe was modified by grinding the probe at an angle to produce a sharp point. This allowed the probe to pierce the foil thus giving a satisfactory sampling of the cup contents. An exact duplicate of the original Buna-N-washers of the tube end fittings and plastic cover plate of the Sampler II were made of polytetrafluoroethylene. This was done to eliminate the adverse effects of chloroform.

The chromatographic components used in the automated column system were LC-½-13 column, CAV valves and metering pump. The design of this equipment was such that it could be connected to form a sequential system which performs several

steps in an uninterrupted manner. These components minimized problems arising in automated chromatography in that all connections and valves had zero dead volume and a uniform diameter flow passage. The pump produced reproducible flow rates thus allowing all operations to be performed on a precise time schedule. The LC-½-13.

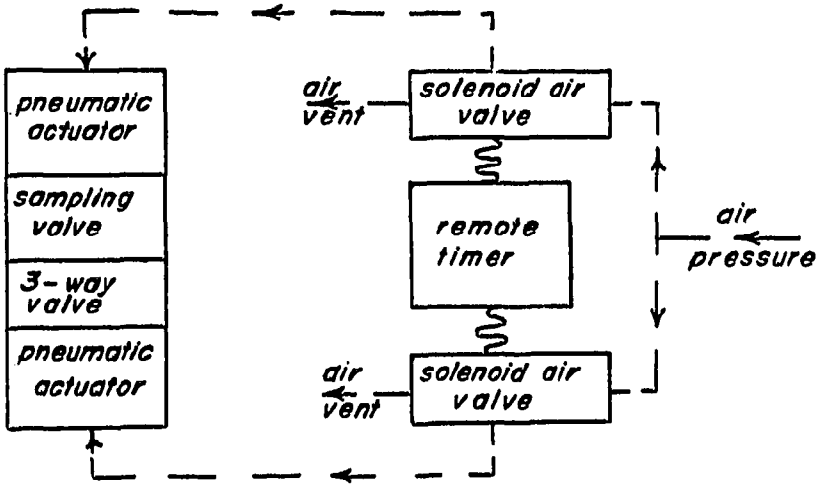

Fig. 4. Modular assembly of automated sample-injection valve.

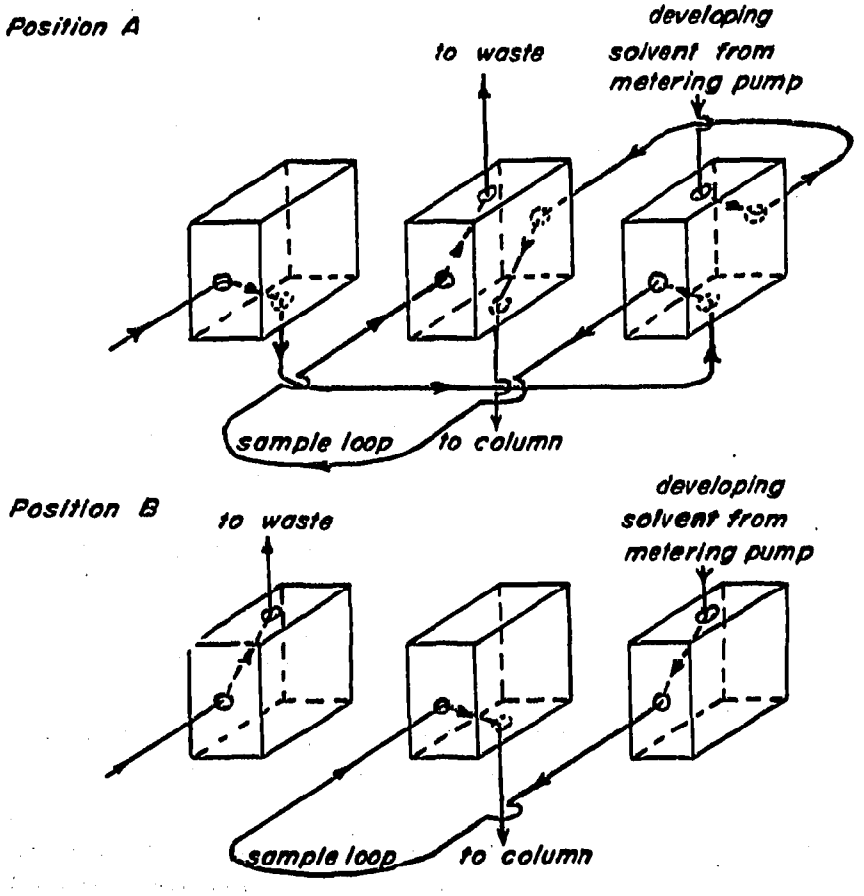

Fig. 5. Schematic liquid flow diagram of the sample injection valve.

column consisted of inlet and outlet plunger assemblies with polytetrafluoroethylene bed supports. The two assemblies were identical except for length. Both assemblies were inserted into the column and adjusted so that the bed of the inlet assembly was at a distance of 12.4 cm from the bed of the outlet assembly. The module design of the automatic-sample-injection valve arrangement and the liquid flow diagram of the valves are schematically illustrated in Figs. 4 and 5. Fig. 4 shows the three-way valve and sample injection valve which were stacked together with a pair of pneumatic actuators, one on each end. The valve parts were connected together with 0.031 in. polytetrafluoroethylene tubing. The actuators were controlled by solenoid-operated air valves which were sequenced through a remote timer box. The flanges of the tubing were firmly pressed against the sides of a slider, which was sequentially moved from one position to the other by means of the air-operated pneumatic actuators. Fig. 5 is a schematic flow diagram of the valve assembly. The squares represent the cross section of the sliders, one in each position. Holes in the sliders connected tubes entering valve ports. The position of the slider determines which tubes were connected: position A, and position B. When the valve was in position A, solvent flowed to the column via the bypass loop while the sample was drawn from the sample cups into the sample loop by means of the proportioning pump. In position B developing solvent flows to the column via the sample loop, thereby forcing the sample ahead of it onto the column. By repeating this procedure a new sample was injected every 5 min without opening the column or stopping the flow. 0.1 ml of the injected sample was determined by the length of the sample loop.

The remote timer was a device which synchronized two functions in the automated system. First it took over the normal timing operations of the Sampler II and then synchronized these operations to those of the pneumatic air valves which control the sampling valve. This device was operated by a gear on a synchronous motor which drove a camshaft through a replaceable gear change rack. The gear ratio of the gear rack provided the cycle time. Two split-design, switch-actuating cams on the shaft were individually adjustable. One cam controlled the sampler while the other controlled the sampling valve.

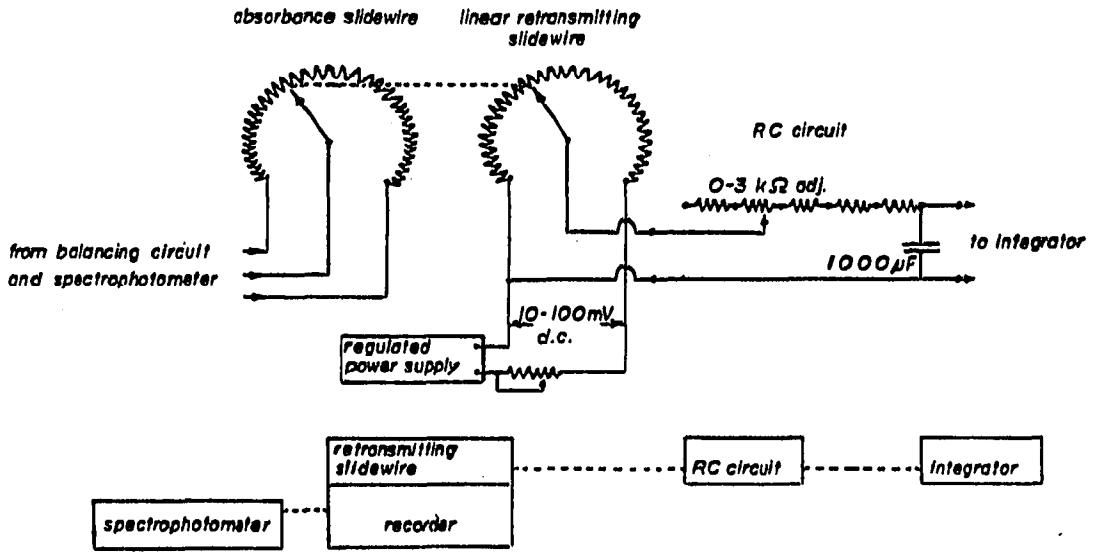

Fig. 6. Schematic diagram of slidewires and RC circuit.

The spectrophotometer signal of the detection system was fed into a strip chart recorder and digital integrator. In order to obtain quantitative results modification of these components was necessary. The recorder, already equipped with a linear absorbance slidewire was modified by installing a linear retransmitting slidewire having its own power. The signal from the linear retransmitting slidewire was fed to an RC circuit modification of the digital integrator. An adjustable RC circuit produced a time constant up to 3 sec to accept the signal from the recorder. Fig. 6 shows a schematic diagram of the slidewires and the RC circuit. The integrator converted the signal into digital form and automatically measured the peak areas generated by the signal as a number which was the printed out area in microvolt seconds.

The method described here for the quantitative determination of tetracyclines is simple and rapid. Work has been done to modify this procedure to determine individual tetracyclines in fermentation-process-control-samples in which mixtures resulted from failure of the strain used to produce a pure tetracycline and also in the study of stability of each individual tetracycline in pharmaceutical formulations.

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