Automated Analysis of Erythromycin

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An automated method has been developed for the analysis of erythromycin by adaptation of the long-established manual U.V. procedure. The method uses a basic automatic analyzer (Technicon AutoAnalyzer) arrangement and a Hitachi Perkin-Elmer spectrophotometer. The method features the concept of the simultaneous determination of a sample and its associated blank in a dual-channel arrangement. A unique cell positioner controlled by a modified liquid sampler enables the use of only one spectrophotometer and one recorder to monitor both channels. In this manner critical phasing problems have been eliminated. The system is capable of making at least 300 measurements per man per day. This method has been developed for use in fermentation broths, process samples, purified materials, and dosage forms.

ERYTHROMYCIN[1] is a broad spectrum antibiotic that has been marketed for many years. Kuzel *et al.* (1) published a spectrophotometric method for the manual determination of this antibiotic. This method is still used routinely in these laboratories. The method is useful for the determination of erythromycin in dosage forms, process samples, and fermentation broths. This manual method is based on a mild alkaline hydrolysis of erythromycin producing a chromophore which has an absorbance peak at 236 mμ. To determine materials other than erythromycin which have an absorbance at 236 mμ, the erythromycin is blanked out by a mild acid treatment. The absorbance difference between the two determinations has been shown to be due to erythromycin alone even in the case of degraded samples or in fermentation broths.

Since sample loads had become increasingly large, it became necessary to consider an automated method. It was decided to attempt automation of the spectrophotometric method because of the long history of reliability of this method. Certain requirements were imposed on the automated method. A 1-hr. service is often required on certain samples, so it became imperative that samples and blanks be determined simultaneously. Second, it was desired to duplicate as much as possible the manual method and this involved contending with high blanks in fermentation samples. The first requirement necessitated the use of either two separate analytical trains or a dual-channel system. The second requirement would tend to eliminate differential spectrophotometry since phasing problems become very serious with two highly absorbing solutions and a relatively low net absorbance. The purpose of this paper is to describe a dual-channel system for the analysis of erythromycin, but more importantly to describe a generalized approach to precise, dual-channel spectrophotometry using only one spectrophotometer and one recorder.

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[1] Marketed as Ilotycin by Eli Lilly and Co., Indianapolis, Ind.

EXPERIMENTAL

Apparatus—The analytical system consisted of the following modules: (*a*) automatic sampler[2] (modified), proportioning pump, and heating baths (modified) from Technicon Instruments, Inc., Chauncey, N.Y.; (*b*) a Hitachi Perkin-Elmer spectrophotometer model 139; (*c*) micro flow cells 9120-NO5, from A. H. Thomas; and (*d*) a Honeywell Electronic 15 recorder (linearized).

Procedure—Figure 1 shows a flow diagram of the automated method including all pump tubing sizes. A sample is drawn from a liquid sampler and is split into two equal portions.

One half of the sample is drawn into the sample channel shown in the lower portion of the figure, and the other half to the blanking channel shown in the upper portion of the figure. The sample channel will be described first. Sodium hydroxide (0.25 *N*) is diluted with water, and the diluted alkali is added to the sample stream, segmented with air, and mixed. The stream is heated in one coil of a 65° heating bath, cooled, and debubbled just before it enters flow cell A of the spectrophotometer.

The other half of the sample is diluted with water, and 0.5 *N* sulfuric acid is added through an H3 fitting. The stream is segmented with air, mixed, and the erythromycin inactivated in the 43° heating bath fitted with 19 ft. of 0.076 i.d. Teflon tubing. As the stream leaves the inactivation bath, 0.35 *N* sodium hydroxide is added to adjust the stream to the same alkalinity as the sample stream. The blank stream enters the second coil of the 65° heating bath, is subsequently cooled and debubbled, and enters flow cell B of the spectrophotometer.

The streams are drawn through the flow cells by pumping a controlled amount from the flow cells while the excess solution and air flow to waste just

[2] AutoAnalyzer Sampler II.

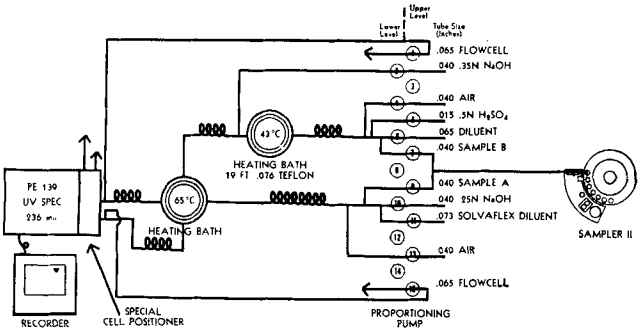

Fig. 1—Flow diagram of the automated method for erythromycin.

ahead of the flow cells. The two channels are alternately monitored in the spectrophotometer at 236 mμ by use of a unique cell positioner. The cell positioner is controlled by a modified liquid sampler.

Standards are run about every 10 or 20 samples, since the stability of the system has been excellent. Reference standard erythromycin base is dissolved in about 10 ml. of methanol and diluted to a concentration of about 250 mcg./ml. with pH 7.0 phosphate buffer. Only one concentration of standard is used since the response of the system is linear. Samples are diluted to about the same concentration as the standard, with the exception of fermentation broths, which are diluted with water so that the maximum absorbance of the sample does not exceed the absorbance range of the analytical system. In these laboratories, this is equivalent to about 20–100 mcg. of erythromycin per ml. of diluted broth. Duplicate cups of sample solutions are generally placed in the liquid sampler, and the results of the duplicates are averaged for calculation purposes. The sampler is operated at 20 samples per hour with a 1:2 sample rinse ratio, so that about 10 samples and blanks in duplicate are processed each hour. Polyoxyethylene (23) lauryl ether[3] is added to deionized water, and this solution is used as the diluent in the automated method.

RESULTS AND DISCUSSION

The automated method follows Beer's law over the entire absorbance range of the system, and the steady state response is very stable. In fact, the steady state has been used to follow long-term hydrolysis curves of several esters of erythromycin in a dynamic system. Figure 2 is a photograph of typical response curves obtained on standards and relatively pure materials. The response at the left of the photograph shows the stable base line obtained and the ability to match flow cell blanks with the cell positioner. The short spikes seen on the base line are the switch points of the flow cells. Peaks 1, 3, and 5 are standards and peaks 6, 8, and 10 are the blanks associated with the standard. Peaks 7 and 9 are the first sample and 12 and 14 are the associated blanks. It is apparent that the blanks are located about midway between sample peaks and are offset from their respective samples by 5 places due to the difference in transit times of the two channels. A time delay coil could be installed in the sample stream to provide adjacent sample and blank peaks if

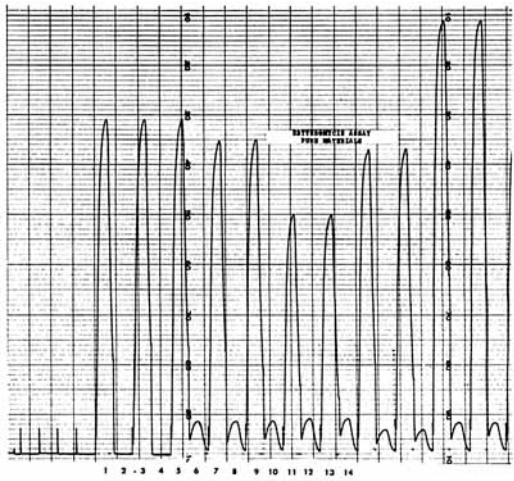

Fig. 2—Recording of replicate samples of erythromycin standards and samples of relatively pure materials illustrating the dual-channel recording technique.

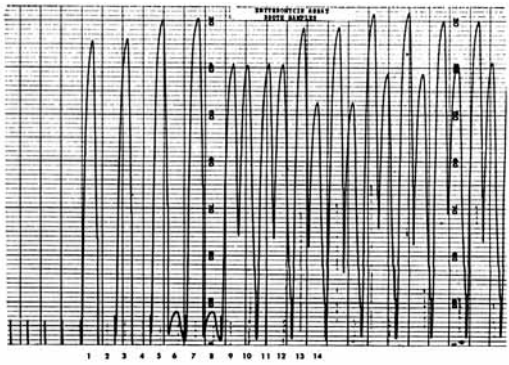

Fig. 3—Recording of replicate samples of erythromycin fermentation broth samples.

desired. The excellent reproducibility of the system is evident in the illustration. There is no sample interaction, although this is not clearly illustrated in Fig. 2 because of the necessity for cell switching. In practice, small changes in phasing occur from day to day and within days; but since only individual peaks need to be observed, rephasing is hardly ever re-

[3] Marketed as Brij 35 by Atlas Chemical Industries, Inc., Wilmington, Del.

TABLE I—COMPARATIVE DATA ON ERYTHROMYCIN SAMPLES

Lot No.	Manual	Automatic	Microbiological
1	938 mg./Gm.	937	970
2	932 mg./Gm.	926	933
3	905 mg./Gm.	907	965
4	930 mg./Gm.	936	960
5	20,600 mcg./ml.	20,300	
6	44,900 mcg./ml.	44,700	
7	2,430 mcg./ml.	2,670	2,180
8	2,100 mcg./ml.	2,180	2,320
9	1,480 mcg./ml.	1,560	1,280

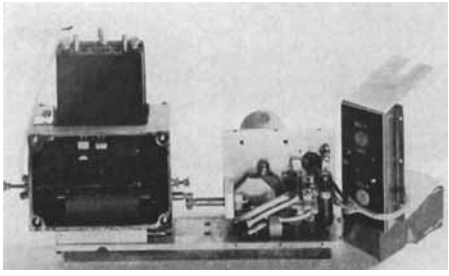

Fig. 4—Interior of the cell positioner.

quired. A water blank is inserted in the sample tray occasionally to assure that no base line drift has occurred. Relative standard deviations of the average of five replicates corrected for blank are typically less than 1%. Occasionally deviations greater than those indicated are observed in the sample readings, but when the associated blanks are subtracted, the relative standard deviations are normal. This may occur because of some contamination of the sample by the sample cup since sample cups are not prewashed in this laboratory.

Figure 3 illustrates typical response curves obtained on fermentation broth samples. Peaks 1 and 3 are standards and 6 and 8 are the respective blanks. Peaks 5 and 7 are the first broth sample and 10 and 12 are the blanks for the first sample. The blank absorbances in this case are much higher, and net absorbances are relatively small. The need for precision in the system is readily evident. This also illustrates the problems that would occur if a differential spectrophotometric method were employed since only slight phasing changes would result in gross errors.

Some comparative results are shown in Table I. Validity of the analysis is demonstrated by comparison with microbiological assays. The first four results are on relatively pure materials, the next two on process samples, and the last three on fermentation broths. The automatic and manual methods are in good agreement, and both closely follow the results obtained by microbiological analysis.

Previous experience in this laboratory with a large number of flow cells has resulted in a distinct preference for the design of the A. H. Thomas flow cell. This flow cell has excellent clearance characteristics, has a small internal volume, and any occasional air bubbles are readily cleared. The authors also wanted to use the Hitachi Perkin-Elmer model 139 spectrophotometer because of its excellent stability and proven performance. The only cell positioner available for this combination of components was the A. H. Thomas cell positioner but, unfortunately, it was not designed to position microflow cells with small cell windows, and large errors were observed in its use due to positioning problems alone. With no other cell positioner commercially available, the authors decided to design and build a precision cell positioner. Figure 4 is a photograph of the interior of the cell positioner that was constructed from this design. Only two positions were required for this application, so a unit was designed that would position between two adjustable stops. The stops can be seen mounted into the ends of the cell compartment. A new cell holder carriage was made to provide a firm grip on the cell holder. The manual cell positioner shaft was removed from the cell compartment and was replaced by a smooth shaft fitted at one end with a ball joint which engaged a pin fastened to the underside of the cell holder carriage. The other end of the shaft was connected by means of a partially threaded pin to the shaft of the cell positioner assembly. The cell holder carriage can be easily uncoupled from the cell positioner and moved manually to one of the other two cell positions, if desired. A Slo Syn motor (SS25 Superior Electric Co., Bristol, Conn.) operates a yoke fabricated from an acetyl resin[4] which in turn is mounted on the sliding shaft of the cell positioner. The yoke drives the shaft until one end of the cell holder carriage contacts one of the adjustable stops and the remainder of the stroke is taken up by a spring located within the arms of the yoke. A similar action occurs on the return stroke. The spring between the arms of the yoke is slidably mounted on the cell positioner shaft and is retained by small horseshoe-shaped keepers. In addition to providing facilities for overtravel of the yoke, the spring also assures a firm reproducible contact with the adjustable stops and reproducible cell positioning. The stops are adjusted from the outside of the cell compartment for proper cell alignment and matching of cell blanks. The Slo Syn motor used in this device caused an annoying vibration in the cell positioner, and the condition was corrected by installation of a 500 ohm 5-w. variable resistor in parallel with the motor. This dropped the voltage applied to the motor to about 50 v.

A cell positioner should be interlocked with the rest of the analytical train so that a slow cumulative drift cannot occur. The positioner just described is controlled by a special modification of the automatic sampler. Figure 5 shows a wiring diagram of the control circuit for the cell positioner. A microswitch was installed on an existing mounting post inside the sampler. This switch is labeled as Technicon sampler switch in the diagram and is actuated by a double rise split cam attached to the shaft of the sampler timing motor. The cam on the timing motor is split so that the ratio of dwell times in positions A and B of cell positioner can be varied, if necessary. As the sampler switch is moved to one position, it completes a circuit through the switch on the cell positioner and through the motor. The motor will drive until a single rise cam attached to the motor shaft opens the cell positioner switch and

[4] Marketed as Delrin by E. I. duPont de Nemours & Co., Wilmington, Del.

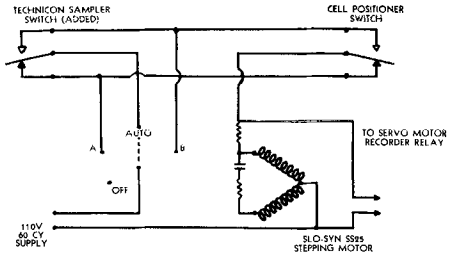

Fig. 5—Wiring diagram of the control circuit for the cell positioner.

thus opens the circuit. This action corresponds to one switching movement of the flow cells. The sampler timing motor continues to run until the double rise cam returns the sampler switch to its former position, whereupon the motor circuit is again closed and the motor drives to its former position, thus completing its switching cycle. Large spikes resulting from temporary blockage of the light path due to switching are prevented from being recorded on the recorder by insertion of an air damped relay into the servo motor circuit of the recorder. The relay is activated every time the motor is energized. The delay can be adjusted to essentially eliminate any spikes without significant loss of meaningful recorded data. The cell positioner can also be operated manually for set-up purposes by use of the A and B position manual switch. The cell positioner as described was designed for a sampling rate of 20 per hour. If a higher sampling rate is desired, the number of raised portions on the split cam mounted on the sampler timing motor must be increased to correspond to the number on the programming cam controlling the sampler.

Approximate phasing of the two channels was accomplished in the usual way by insertion of a time delay coil in one of the channels. The cell positioner must then be phased with the sampler so that cell positioning is achieved at the appropriate time. This was accomplished by designing a new programming cam for the sampler. A double rise split cam was fabricated from the acetyl resin and both halves of the split cam can be rotated with respect to the center hub of the cam which is driven by the timing motor. Adjustment of the split cam provides for a variable sample to rinse ratio. Rotation of the adjusted split cam on the hub provides for advance or delay of sampling with respect to the double rise cam controlling the cell positioner and in effect controls the time span between sampling and cell positioning.

The absorbance of the solutions in both channels is recorded on a Honeywell Electronic 15 strip chart recorder equipped with a 1 cycle log-characterized slide wire and having a range of 0 to 1 absorbance unit. This recorder provides a very precise and reliable recorder for spectrophotometric work at moderate cost. Linear potentiometric recording cannot be done on the instrument modified in this manner; but since this recorder was to be used for spectrophotometric work only, this limitation is not serious.

The lengths of the various heating coils, the temperatures of the baths, and the concentration of reagents were all experimentally determined to substitute for the conditions found in the manual method. A 9-min. acid inactivation of the erythromycin at 43° with 0.5 *N* sulfuric acid was found to be equivalent to the 1-hr. inactivation at room temperature with 0.25 *N* sulfuric acid as performed in the manual method. A plateau between 38 and 48° was found for the inactivation temperatures. Any temperatures above or below these figures produce high and invalid blanks.

Certain special precautions need to be observed in the use of this system. Because of the low wavelength, *i.e.*, 236 mμ, considerable and variable extraneous U.V. absorbance can be picked up from some lots of Tygon tubing especially in the presence of warm alkaline solutions. Teflon, polyethylene, or glass tubing was used throughout the system except for the pump tubings. The cell compartment was fitted with an A. H. Thomas high rise cell cover and the cells were connected to the debubbler fitting with thin wall flexible polyethylene tubing. Relative dilutions in the two channels are important in a dual-channel system, especially where one channel is a blanking channel having high absorbance. In an attempt to determine relative dilutions, erythromycin was run as a sample in both channels, but subsequently it was found that some erythromycin was destroyed in the extra heating step of the blank channel. A stable compound having U.V. absorbance at 236 mμ was needed, and sodium benzoate was found to work very well for this purpose. The pump tubings are adjusted until both channels provide the same dilution of the sample. The model 139 spectrophotometer has a very rapid response, and somewhat noisy tracings were initially obtained. By using the spectrophotometer in its slow response mode, very smooth response curves were obtained without any loss in precision.

ANALYTICAL POTENTIAL

Aside from the use of this method for the analysis of erythromycin, this paper describes a general method of monitoring two analytical streams for both U.V. and colorimetric methods while using only one spectrophotometer and one recorder. This was made possible by the design of a precise cell positioner obviating the need for differential spectrophotometry and its attendant phasing problems. The unique design of the cell positioner also permits the use of microflow cells in a cell positioner suitable for use with a Hitachi Perkin-Elmer spectrophotometer. The cell positioner design could easily be adapted for use with other spectrophotometers. The concept of a cell positioner controlled by the modified automatic sampler could be utilized in a number of other applications.

REFERENCE

(1) Kuzel, N. R., Woodside, J. M., Comer, J. P., and Kennedy, E. E., *Antibiot. Chemotherapy*, 4, 1234(1954).