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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF KANAMYCIN*

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SUMMARY

A fast, selective, and precise liquid chromatographic method for simultaneous, independent determination of kanamycins A and B is described. Sample components are separated on a pellicular cation exchanger and monitored by fluorescence using post-column on-line derivatization. Less than 0.35 μg of kanamycin B can be detected in as much as 7 μg kanamycin A injected. The detection limit for kanamycin A is less than 20 ng injected. Reproducibility of the entire chromatographic system is about 1% (2 σ) based upon repeated injections of standards. Precision of repeated process sample preparations is about 6% (2 σ). Chromatographic analysis time is less than 15 min per sample.

INTRODUCTION

The general term kanamycin includes at least three known aminoglycosides shown in Fig. 1. Kanamycins A and B are fermentation produced antibiotics, registered and sold in several countries. The fermentation of these separate compounds is quite selective; even so, the need to monitor one in the presence of the other may arise. The structurally similar kanamycins are difficult to differentiate by chemical or biological tests.

Our laboratories needed an analytical method for process streams to quantitate kanamycin A without interference from kanamycin B present, and further to quantitate kanamycin B if present at levels greater than about 5% of the kanamycin A content. A large number of samples were to be tested. Normal biological methods were unsuitable because of unequal response and mutual interference to test organisms. A number of chemical tests have been reported for kanamycin in the literature but none appeared suitable for this purpose.

The large number of samples to be processed limited our choice of methods to separations which could be run in parallel or to sequential tests which required less than about 10–15 min per sample. A number of possibilities were considered including several forms of chromatography. Thin-layer and paper chromatography were ruled

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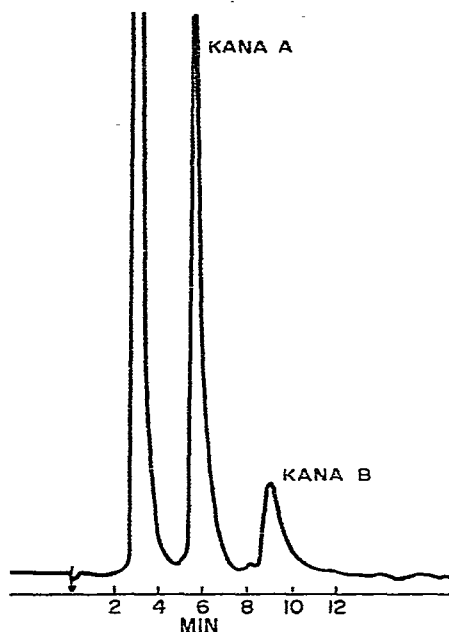* Presented at the National ACS Meeting, Chicago, Ill., August, 1975.
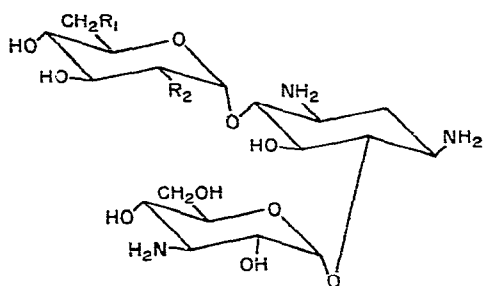
Fig. 1. Structure of kanamycins.

Fig. 2. High-performance liquid chromatographic separation of kanamycins A and B on a strong cation exchanger. Column, 1 m × 2 mm Zipax SCX (37–44 μ). Temperature, 23°. Detection, refractive index. Mobile phase, 0.01 M (NH$_4$)$_3$PO$_4$ (pH 9.1).

out on the general basis of poor precision. Gas chromatographic analysis has been reported by Tsuji and Robertson[1]. We have also had some experience with gas chromatography of kanamycin. In general we find that with the multiple functional groups to be derivatized and at the high column temperatures required, precision is not very good. We have obtained a better separation of kanamycins A and B than reported by Tsuji and Robertson, but we have been unable to achieve quantitative results with kanamycin B. Processing aqueous samples is an added complication.

Kanamycins have been separated by column chromatography since 1958 when Rothrock et al.[2] used a 4-ft. section of 1-in. pipe to separate kanamycins A, B, and C on a strong anion exchanger. This procedure was improved by Inouye and Ogawa[3] using a 0.9 × 39 cm column of 200–400 mesh strong anion-exchange resin. Baseline separation was obtained among kanamycins A, B, and C in 3½ h, kanamycin B eluting first. An AutoAnalyzer®–ninhydrin system was used for on-line monitoring. This test was adopted by the British Pharmacopoeia Commission[4] who described a fraction collector and a manual ninhydrin test for detection. The same basic separation was performed by Ottake and Yaguchi[5] on a 1 m × 2 mm microreticular anion-exchange resin (Aminex A-27, 7–11 μm particle size) in the hydroxyl form. A Varian Aerograph Model 4200 liquid chromatograph was used with a refractive-index (RI) monitor. The entire chromatogram required about 18 min. Using our own slurry-packed anion-exchange columns we were able to simulate the Varian chromatogram but were unable to improve on it. Nevertheless liquid chromatography (LC) appeared to hold promise for a rapid, and precise method for individually determining kanamycin A and B in complex samples.

We therefore began to study the behavior of kanamycins on other chromato-graphic supports. Because of the ionizable amine groups of the kanamycin molecules, cation exchange seemed feasible. A very good separation of kanamycin A and B was obtained using the refractive-index monitor (Fig. 2). However, refractive-index monitoring was not suitable for routine testing because of noise, drift, and poor sensitivity to the sample compounds.

Since kanamycins do not have suitable ultraviolet (UV) absorption, some other means of monitoring was required. Derivatizing the sample either before or after chromatography was considered. Because of the multiplicity of reactive functional groups and the need for reproducible, quantitative derivatization for pre-chromato-graphy derivatizing, we decided to attempt post-column on-stream derivatizing.

Post-column fluorimetric detection was successful and this report describes the high-performance liquid chromatographic system developed for rapid, precise analysis of kanamycins A and B in complex process samples. At the time this work was completed it was the first to our knowledge to use on-line fluorescence deriva-tization for monitoring chromatograms requiring about 5–15 min.

EXPERIMENTAL

Equipment

Several LC systems were used in the development work. All were modular, assembled from commercial components (Fig. 3). The mobile phase and reagent reservoirs were erlenmeyer flasks suitably protected from the environment and main-tained at ambient temperature. Pumps were Milton Roy Minipumps (Model 19-60066-001). Various types of commercial pressure gauges and valves were used. Pressure gauges also served as suitable pulse dampers. Both Varian stop-flow injectors (Model 02-001652-00) and Chromatronix valve injectors (Model HPSV-20) were used. Columns were either 1 m or 0.5 m × 2.1 mm I.D. 316 stainless steel with a 10-μm Swagelok snubber for the end fitting. The reagent line joined the column effluent via a 1/16-in. Swagelok Union Tee (Part No. SS-100-3). A 10-ft. length of Chromatronix Cheminert tubing (0.012 in. I.D.) at ambient temperature served as the reaction coil. The LDC Model 1309 fluoromonitor with a 13-μl quartz spiral-tube
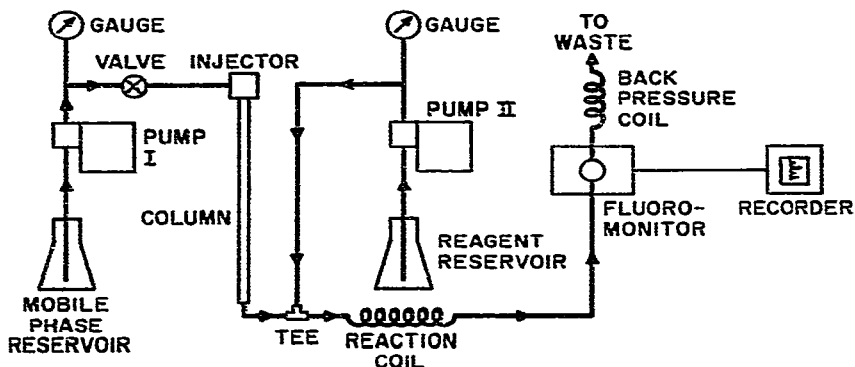

Fig. 3. LC system for kanamycin using fluorimetric detection.

flow cell was used with either a Hewlett-Packard Model 7127A or a Heath Schlumberger Model SR255B strip chart recorder. A back-pressure coil of 10 ft. × 0.012 in. I.D. tubing was attached to the monitor exit line to reduce bubble problems.

Reagents and chemicals

Water was distilled and de-ionized. Kanamycin standards were Bristol Labs. Control Reference Materials. o-Phthalaldehyde (o-PTH) (Cat. No. P3940-0), also referred to as o-phthalicdicarboxaldehyde, and 2-mercaptoethanol (Cat. No. M370-1) were obtained from Aldrich. Fluorescamine was Fluram® (Hoffman-LaRoche, Cat. No. 43023). All other chemicals were ACS reagent grade and used as received from the vendor.

Chromatographic procedure

Empty columns were sequentially washed with methylene chloride, acetone, water, and acetone, and air dried. Columns were then dry-packed by the method of Kirkland[6] with a pellicular cation-exchange resin (DuPont Zipax SCX, Cat. No. 8209500002 or Whatman HS Pellionex SCX). The mobile phase was prepared to contain 0.01 M ethylenediamine tetraacetic acid (EDTA) adjusted in the pH range 9.0–9.5 with potassium hydroxide and pumped at a flow-rate of approximately 1 ml/min. The fluorimetric reagent was prepared to contain either 15 mg of fluorescamine in 100 ml of dry acetone or 80 mg of o-PTH, 0.02 ml 2-mercaptoethanol, and 1 ml ethanol in 100 ml of pH 10 borate buffer (0.01 M). The fluorimetric reagent was pumped at a flow-rate of 0.5 ml/min. Columns were conditioned overnight at normal flow-rates.

Reference standards and samples were prepared in water at concentrations of 200–400 μg/ml. Kanamycin B reference standard was prepared at concentrations as low as 40 μg/ml. Aliquots (20 μl) were applied to the columns. Peak areas were measured as the product of height and half-width and samples were calculated from standards by direct proportionality without the use of internal standardization.

RESULTS AND DISCUSSION

Optimization of chromatography

A number of pellicular strong cation exchangers were used during methods development, including Zipax SCX, Vydac SCX, and HC and HS Pellionex SCX. Generally the packings performed similarly although slightly better efficiency was obtained with Zipax SCX than with the bonded phases. Efficiency deteriorated with column use for all of the packings. Under conditions used for the assay, the efficiency of new columns was anywhere between 200 and 500 plates per meter and deteriorated to less than 100 plates per m over 2–4 weeks use. A typical separation of A and B on a column having about 150 plates per m is shown in Fig. 4. One of the best chromatograms of an actual process sample is shown in Fig. 5. The kanamycin peak has a plate height of about 2 mm.

A number of mobile phase parameters were tested to optimize the separation in the shortest time. The most difficult separation was A and B, particularly since both tailed and A, the major component, eluted first. But these two components also had to be separated from other sample constituents eluting near the void volume. Increasing
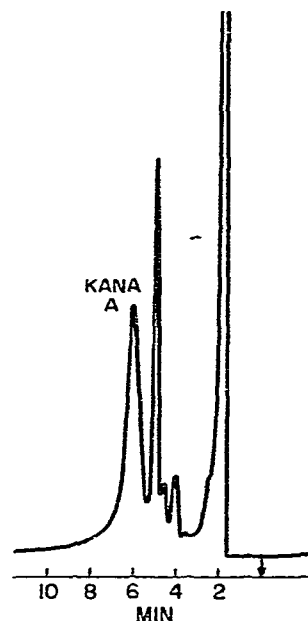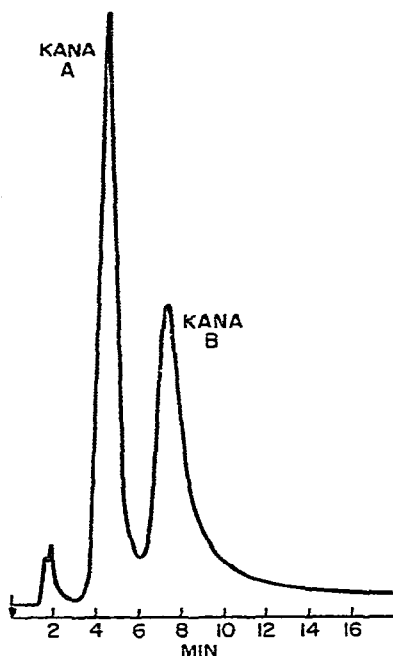
Fig. 4. Typical chromatogram showing separation of kanamycins A and B at high levels. Column, HS Pellionex SCX. Detection, fluorescence–o-PTH. Mobile phase, 0.01 M K^+ EDTA (pH 9.5).

Fig. 5. Chromatogram of kanamycin process sample. Columns, 1 m × 2 mm Zipac SCX (37–44 μ). Detection, fluorescence —o-PTH. Mobile phase, 0.01 M K^+ EDTA (pH 9.3), 0.8 ml/min. HETP, 2 mm.

ionic strength or pH caused both kanamycins A and B to elute earlier, but pH affected B more than A. Thus, these two parameters were adjusted to obtain suitable retention times when column length or flow-rates were changed. Kanamycin C eluted near the void volume under all conditions.

The type of cations (NH_4^+, K^+, Na^+) or anions (SO_4^{2-}, PO_4^{3-}, $C_2H_3O_2^-$) in the mobile phase did not seem to change the separation except that no separation was obtained using borate, which perhaps complexes the kanamycins as well as other sugars. Ammonium ions and ethylenediamine tetraacetate ions both reduced tailing. Ammonia was incompatible with the fluorescence reagents and could not be used. The addition of secondary or quaternary amines to the mobile phase had no apparent effect on tailing. Addition of a very low concentration of kanamycin B (about 10^{-5} M) to the mobile phase improved the response and peak shape of low levels of kanamycin B injected, at the expense of increased noise in the system. Neither added solvents nor elevated temperature improved peak efficiencies.

For routine use, pH and flow-rate were adjusted to give kanamycin A at a 5–6 min retention time and kanamycin B at about 8–10 min. The entire chromatogram required about 11–13 min, of which only 10–15 sec was contributed by the reaction coil of the detection system.

Optimization of detection system

Kanamycins do not have suitable UV absorption and for our initial work we

used an RI monitor (LDC Model 1107). In our hands the RI monitor was too sensitive to the environment and insufficiently sensitive to kanamycin (the detection limit was about 5 μg kanamycin B). To obtain better sensitivity we attempted colorimetric reactions. Ninhydrin was used on-stream but the reaction required several minutes of heating and too much resolution and time were lost. Trinitrobenzene sulfonic acid[7] in a 1–2-ml holding coil at 80° was also used on-stream for several months. An LDC
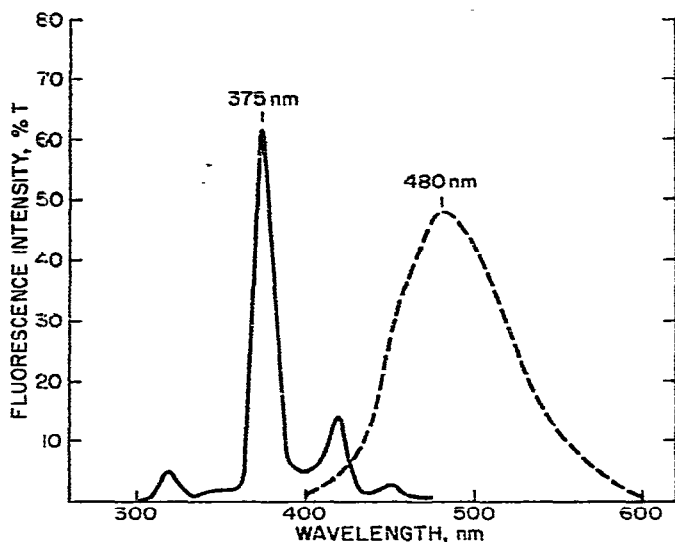

Fig. 6. Excitation and emission spectra of kanamycin. Fluorescamine reaction. Kanamycin, 1.02 μg/ml; pH 8.6. Meter multiplier, × 0.3. Kanamycins A and B coincide. ———, Excitation; — — —, emission.
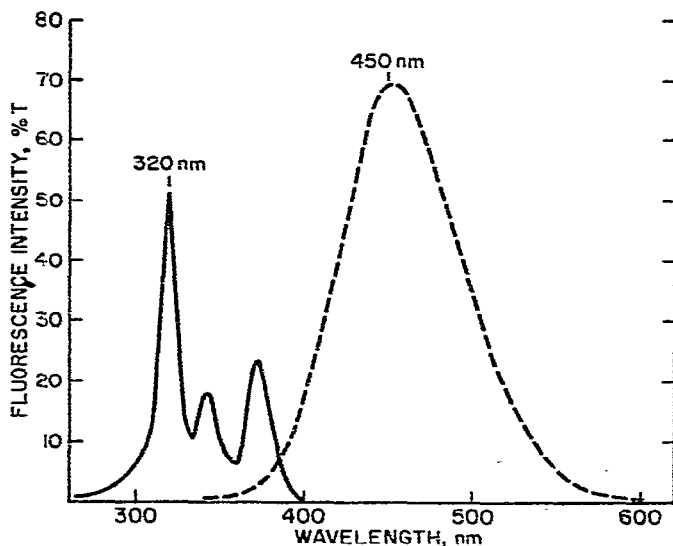

Fig. 7. Excitation and emission spectra of kanamycin. o-PTH reaction. Kanamycin, 2.79 μg/ml; pH 9.0. Meter multiplier, × 1.0. Kanamycins A and B coincide. ———, Excitation; — — —, emission.

Model 1205 UV Monitor with a 440-nm filter was used as a detector. Sensitivity for kanamycin was improved (less than 1 μg injected could be detected) but the system was plagued with bubble formation and noise. Although these problems might have been overcome, we decided to study the fluorescamine reagent published by Udenfriend et al.[8], Stein et al.[9], and Böhlen et al.[10]. This reagent was reported to react very rapidly with amines at room temperature to yield highly fluorescent derivatives.

Because of the cost of fluorescamine, the o-PTH reagent for amines first published by Roth[11] and Roth and Hampai[12] was also investigated in the flow system. Both reagents worked equally well. The o-PTH reagent has the advantage of solubility and stability in an aqueous carrier, which minimizes problems of bubbles, precipitation, and pH control. A number of recent papers have reported use of o-PTH[13–15]. The use of fluorescamine, of course, has been even more widely published.

Generation of fluorescence from kanamycin was studied manually using an Aminco Bowman spectrophotofluorimeter, as a function of wavelength, time and pH. The excitation and emission wavelength spectra for the fluorescent derivatives are shown in Figs. 6 and 7. The optimum excitation and emission wavelengths with fluorescamine are 375 and 480 nm, respectively. The o-PTH spectra show three excitation maxima and an emission maximum at 450 nm. The LDC fluoromonitor has broad excitation with maximum energy at 360 nm. Clearly the proportion of energy at 375 nm is critical for sensitivity to the fluorescamine derivative. At optimum wavelengths both fluorescamine and o-PTH derivatives are approximately linear with concentration (Fig. 8).

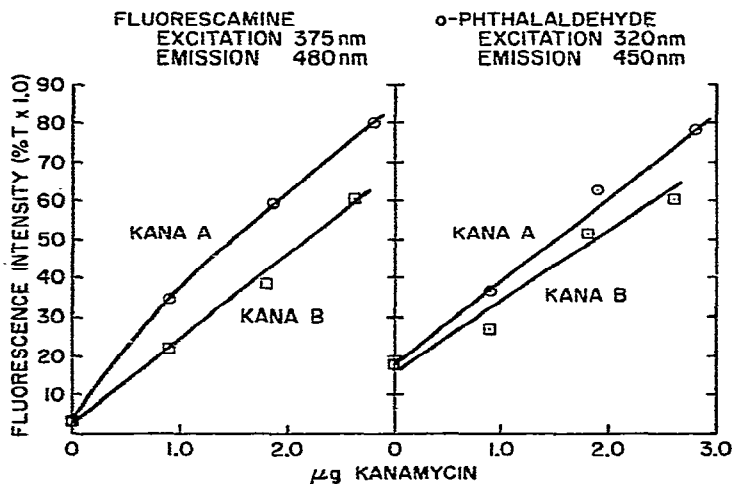
Fig. 8. Manual standard curves of kanamycins A and B with fluorescamine and o-PTH.

Fluorescamine gives lower blank readings and more stable derivatives than o-PTH. The particular batch of o-PTH we used gave a background fluorescence equivalent to about 2 μg of kanamycin. Another batch from a different supplier exhibited much higher background fluorescence. The fluorescamine derivatives show no loss in fluorescence over 30 min whereas the o-PTH derivatives decay exponentially, losing about 30% of the fluorescence intensity in 10 min. (Fig. 9). This is not
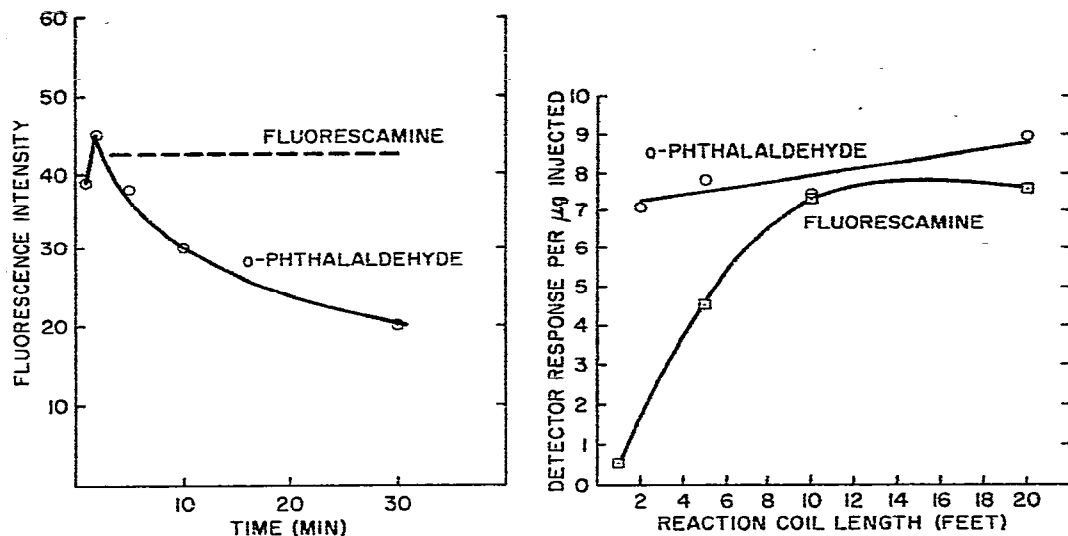
Fig. 9. Rate of fluorescence decay of fluorescamine and o-PTH derivatives of kanamycin A.

Fig. 10. Fluorescence response as a function of reaction coil length. Sample, $10\,\mu g$ kanamycin A. Flow-rates; mobile phase, 1.0 ml/min; reagent, 0.5 ml/min. Reaction coil, 0.012 in. I.D. Cheminert® tubing.
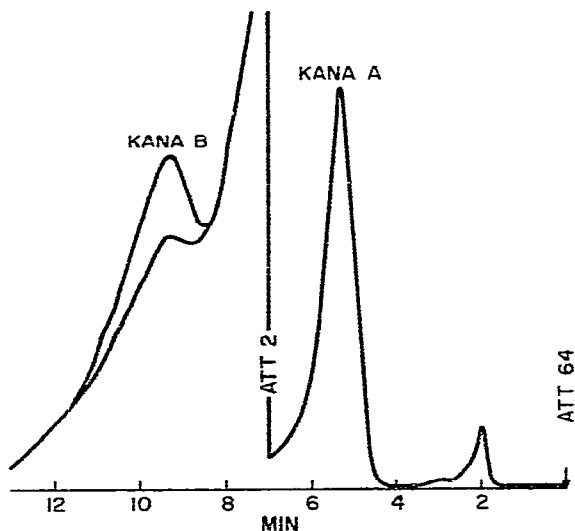

Fig. 11. Chromatograms showing the detection limit of kanamycin B in kanamycin A. Sample, kanamycin A ($7\,\mu g$) + B (0.7 or $0.35\,\mu g$). Column, 1 m × 2 mm Pellionex SCX HS. Detection, fluorescence —o-PTH. Mobile phase, 0.01 M EDTA (pH 9.5), 1 ml/min.

detrimental in the LC system where timing is controlled and reproducible. Equivalent fluorescence intensities were obtained with both reagents over the pH range of 6.5–10 and all reaction rates were very high.

Length of the derivatization reaction coil was studied for each reagent (Fig.10). Response began to plateau at about 10 ft. × 0.012 in. I.D. tubing for Fluram. Even

though a somewhat longer coil gave greater response for o-PTH a 10-ft. coil was used.

Reagent flow-rates were maintained at about 0.5 ml/min. When using o-PTH at higher flow-rates, the response dropped off rapidly: although the ratio of reagent to sample was greater, the resulting derivative was further diluted. At 0.25 ml/min response was slightly less than at 0.5 ml/min.

Fluorescamine was used in the flow system at 15 mg per 100 ml of acetone. At 10 mg per 100 ml a 10% loss in response was noted. o-PTH was used at 80 mg per 100 ml of buffer. Using 40 and 20 mg per 100 ml caused an approx. 12 and 25% loss in response, respectively. Concentrations of 2-mercaptoethanol from $\frac{1}{2}$ to 4 times that specified showed no more than a 10% change in response.

Using the o-PTH detection system, adding kanamycin B to the mobile phase, and substituting a 40-μl quartz tube for the spiral flow cell, the detection limit of kanamycin A or B was less than 20 ng injected. Chromatograms obtained had a good signal-to-noise ratio and substantial area but the peaks were very broad. Considerably lower levels could have been detected if the peaks were narrower.

Using the small flow cell, and in the presence of about 7 μg of kanamycin A, the detection limit for kanamycin B was about 0.35 μg (Fig. 11). The limiting factor for detection of kanamycin B was the peak shape of kanamycin A, the tail of which was exaggerated by attenuating to enlarge the kanamycin B peak.

Validation of the method

The coefficient of variation (2 σ) of repeated injections of standards or samples has been as good as 1%. Reproducibility of standards injected among samples in a run is about 2–3%. The coefficient of variation (2 σ) of separate preparations of the same process sample is about 6% for kanamycin A at a level of about 200 μg/ml (4 μg injected).

Response of kanamycin A is linear over the range 2.5–10 μg injected (the common level of samples) using either fluorescamine or o-PTH. As the amount injected is decreased to 1.0 and 0.1 μg the response holds approximately linear if standards are prepared in the mobile phase. If the standards are injected from water, however, the response to kanamycin actually increases at lower levels. This phenomenon is not presently understood.

Process samples were spiked with kanamycin A and recovered as shown in Table I. On the whole recoveries were good, showing that the method is applicable

TABLE I

RECOVERY OF KANAMYCIN A IN A SPIKED PROCESS SAMPLE

Kanamycin spike (μg/ml)	Kanamycin found (μg/ml)	Spike recovery (%)
0	17.0, 15.4	—
10.6	28.5	116
53.2	71.0, 69.4	103, 100
106	118, 118, 125	96, 96, 102
213	224, 209, 219, 233	98, 91, 95, 102
426	432, 434	98, 98
		Mean 99.6%

over a broad range. Process samples were assayed for kanamycin by the LC procedure and by a biological turbidimetric method. A good positive correlation was obtained on about two dozen samples over several weeks. The LC assay was approximately 6 % lower than the biological assay. Whether this difference was due to small amounts of kanamycin B present, possible interferences in the biological assay, or some other factor was not determined. The samples studied did not show measurable amounts of kanamycin B by LC.

CONCLUSIONS

An LC system has been developed and routinely operated on a 3-shift operation to independently monitor kanamycins A and B in complex process samples. Post-column on-stream derivatization is used to produce fluorescent derivatives for sensitive detection. Good precision and accuracy are obtained in less than 15 min per sample. As little as 20 ng of kanamycin can be detected. The detection system has been further applied in these laboratories to the LC of other primary amino-function-containing antibiotics such as ampicillin and amikacin.

ACKNOWLEDGEMENT

REFERENCES

1 K. Tsuji and J. H. Robertson, *Anal. Chem.*, 42 (1970) 1661.
2 J. W. Rothrock, R. T. Goegelman and F. J. Wolf, *Antibiot. Annu.*, (1959) 796.
3 S. Inouye and H. Ogawa, *J. Chromatogr.*, 13 (1964) 536.
4 *British Pharmacopoeia*, 1973, pp. 238 and A98.
5 T. Ottake and M. Yaguchi, *Liquid Chromatography, Applications Bulletin No. 5*, Varian, Palo Alto, Calif., 1973.
6 J. J. Kirkland, *J. Chromatogr. Sci.*, 7 (1969) 7.
7 D. M. Benjamin, J. J. McCormack and D. W. Gump, *Anal. Chem.*, 45 (1973) 1531.
8 S. Udenfriend, S. Stein, P. Böhlen and W. Dairman, *Science*, 178 (1972) 871.
9 S. Stein, P. Böhlen, J. Stone, W. Dairman and S. Udenfriend, *Arch. Biochem. Biophys.*, 155 (1973) 203.
10 P. Böhlen, S. Stein, W. Dairman and S. Udenfriend, *Arch. Biochem. Biophys.*, 155 (1973) 213.
11 M. Roth, *Anal. Chem.*, 43 (1971) 880.
12 M. Roth and A. Hampai, *J. Chromatogr.*, 83 (1973) 353.
13 S. Taylor and A. L. Tappel, *Anal. Biochem.*, 56 (1973) 140.
14 R. Hakanson, A. L. Ronnberg and K. Sjolund, *Anal. Biochem.*, 59 (1974) 98.
15 J. R. Benson and P. E. Hare, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 619.