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HIGH-SENSITIVITY RADIOASSAY OF CHROMATOGRAPHIC EFFLUENTS

AUTOMATIC FRACTION COLLECTOR/CONCENTRATOR FOR QUANTITATIVE AUTORADIOGRAPHY

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SUMMARY

We modified an automatic micro-fraction collector for fractionating the effluent of a high-pressure chromatography column and concentrating the fractions for radioassay by autoradiography. A succession of aliquots of up to 300 μl of effluent is deposited in wells formed in non-wetting fluorocarbon film. After evaporation to near dryness, the remaining droplets are quantitatively transferred to filter paper where they form uniform spots 2–3 mm in diameter. The paper is then exposed to photographic film for a time determined by the radioactivity of the sample. The film is then developed and the density of the radioautograph scanned.

Most of the resolution of high-performance liquid chromatography (HPLC) was preserved. In HPLC of amino acids with a flow-rate of 1 ml/min, we collected fractions of 125 μl every 7.5 s. The proline peak, which the UV detector measured 21 s wide at half-height, was 23 s wide at half-height on the radioactivity record. After 6 h of radioautography, 1000 dpm of ^{14}C in a 3-mm diameter spot produced approximately 0.2 absorbance units above film background. Absorbance was proportional to radioactivity from 160 to 2500 dpm. With 1 week of exposure, absorbance was proportional and radioactivity could by quantified in spots containing from 5 to 80 dpm.

INTRODUCTION

To increase the convenience, accuracy, and precision of the radioassay method for high-performance liquid chromatography (HPLC) or other chromatographic effluents we described in 1984[1], we have adapted a commercially available automatic micro-fraction collector for use with the system. This paper describes the construction and mode of operation of this device and the analytical results we obtained with it.

EXPERIMENTAL

Materials

^{14}C-Labeled amino acids were purchased from New England Nuclear (Boston,

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MA, U.S.A.); unlabeled amino acids from Sigma (St. Louis, MO, U.S.A.); Whatman No. 1 Chromatography Paper, 0.16 mm thick, from Whatman (Clifton, N.J. U.S.A.); XAR-5 X-ray film from Kodak (Rochester, NY, U.S.A.); and glycerol from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Equipment

HPLC was performed with an Altex 110A pump (Altex Scientific, Berkley, CA, U.S.A.); equipped with a Rheodyne 7120 injection valve. The analytical columns, packed with Nucleosil C_{18} by the downward slurry technique, measured 10 × 0.42 cm I.D. for analysis of pipecolic acid and 15 × 0.42 cm I.D. for analysis of proline and hydroxyproline. The ultraviolet absorbance of the column effluent was monitored at 234 nm with a Technicon Fast LC-Spectrophotometer (Technicon, Tarrytown, NY, U.S.A.). The amplified detector signals were read out on a Model 4416 Data System (Nelson Analytical, Cupertino, CA, U.S.A.) and a Model 56 Chart Recorder Perkin-Elmer, Norwalk, CVT, U.S.A.).

Fraction collector

As described in our previous paper[1], samples were collected from the effluent of an HPLC column by an adaptation of the procedure described by Fenimore and Davis[2] for spotting samples on thin-layer chromatography (TLC) plates. With the "contact spotter" devised for this procedure, many samples contained in relatively large volumes of volatile organic solvents are simultaneously concentrated almost to dryness on the surface of a polytetrafluoroethylene film, coated with a fluorocarbon liquid, which is vitually non-wetting. The remaining volumes are then simultaneously and quantitatively transferred to a TLC plate as spots well below 1 mm in diameter. The contact spotter consists of a hollow, rectangular stainless-steel hot-plate. Its upper surface contains two rows of 8 mm diameter depressions, 12 mm apart, center-to-center, and approximately 3 mm deep. At the bottom of each depression is a pin-hole for applying a vacuum. The strip of poly-fluorocarbon film coated with fluorocarbon liquid is placed over the rows of depressions. When vacuum is applied to the hot plate, the film indents into wells at each depression. Samples dissolved in up to 250–300 μl of any of a variety of solvents are deposited into the wells, the plate is covered and the solvents evaporated by heating the plate to 50°C and applying a stream of nitrogen. Because of the fluorocarbon coating, the remaining solvent samples assume an almost spherical shape, which they retain through evaporation. When only a small quantity of solvent remains in the bottom of each well, a quantity that is controlled by addition of a specified concentration of a less volatile liquid to the solvent, the TLC plate is placed over the wells. The vacuum beneath the film is then replaced by a pulse of pressure greater than atmospheric which everts the wells and transfers the samples to the plate. Up to 30 samples can be spotted simultaneously. The entire process of evaporation and spotting, which results in quantitative transfer of material and quite uniform spots, requires 5–10 min to perform with organic solvents as volatile as dichloromethane. Buffered aqueous solutions require somewhat more time.

The feasibility of using the "contact spotter" for preparing fractions of an HPLC effluent for radioassay by autoradiography was demonstrated by attaching a 25 cm long, 0.3 mm I.D. Teflon® tube to the outlet of the HPLC detector flow cell

and mounting its other end over the contact spotter. To increase the practicality of the approach, the procedure was mechanized, using a reconstructed Gilson Micro Fractionator (Rainin, Woburn, MA, U.S.A.). This device uses a rectangular tray, 20.8 cm × 6.4 cm, containing 80 sample cups, in which the dispenser needle deposits approximately 0.5-ml aliquots, which are metered by adjusting either the time over each cup or the droplet count. For purposes of the present study, a "contact spotter" hot-plate was constructed identical in rectangular dimensions and distances between samples to the tray of the Gilson Fractionator. As in the "contact spotter" described by Fenimore and Davis, the depressions were milled 0.9 mm deep on the surface of a 0.6-mm thick aluminum plate and provided with 1-mm holes at their apices. The surface of the plate was polished to mirror smoothness for good contact with the Teflon film. The plate was cemented to a silicone rubber gasket, 1 mm thick, which in turn was cemented to a second aluminum plate, 1.5 mm thick, provided with fittings for applying vacuum to the cavity between plates. A 20 cm long, 3.75 cm wide, 100-W strip heater (Rama Industrial Heater Co., San Jacinto, CA, U.S.A.) was fixed to the bottom plate.

Procedure

Fluorocarbon-coated sample wells were formed by placing a sheet of 0.025 mm thick flurocarbon film over the plate and applying a vacuum. The dispenser needle was connected to the HPLC detector flow cell outlet with 25 cm × 0.3 mm I.D. Teflon tubing. Aliquot size was generally controlled by metering from 8 to 13 drops which provided from 120 to 150 μl (10 fractions per min or per HPLC peak). Timed fractionation was also possible with this device. Up to 300 μl of aqueous solution could be deposited in each of the 80 wells. The volume of residue after evaporation was controlled by adding glycerol (500 μl/l) to the mobile phase. When transferred to filter paper, these residues produced spots 1–2 mm in diameter.

An autoradiogram of the paper was prepared by clamping the paper sheet against X-ray film in a Wolf X-ray cassette, 20 × 30 cm, and incubating it in a freezer at −15°C. The film was then developed, and densitometry was performed with the Model CS-910 thin-layer chromatography scanner (Shimadzu, Columbia, MD, U.S.A.). The scanner record was processed on an HP-3390A integrator (Hewlett Packard, Avondale, PA, U.S.A.).

RESULTS

Proportionality of autoradiogram darkening to ^{14}C

Graded quantities of [^{14}C]proline, autoradiographed for different periods of time produced film darkening proportional to applied radioactivity over a 15–20 fold range of concentrations. Exposure of the film for increasing time periods gave the expected increase in darkening. The film response was proportional to ^{14}C at levels below those that produced film absorbance of 0.4 absorbance units above film background. The response decreased somewhat with radioactivity above that level. (In these experiments, unexposed film yielded 0.5 absorbance compared to air.)

The widest linear range, from 250 to 5000 dpm, was obtained after 6 h exposures. With exposure of 18 h, ^{14}C from 80–1250 dpm/spot could be measured. Extending exposure to 72 h permitted measurement of from 5–80 dpm/spot.

Reproducibility

Three standard aliquots of [^{14}C]proline, 39, 78, and 156 dpm, were assayed with each chromatographic run. In five such assays, the film densities produced had coefficients of variation of 1.9, 4.1 and 6.7%, respectively.

Resolution and recovery

The resolution of the HPLC was largely preserved with the post-column, post-UV detector fraction collection system. Comparison of the UV and radioactivity records of the analysis of a standard mixture of three radioactive amino acids showed similar ability and inability to separate the components of the mixture completely (Fig. 1). In addition to radioactivity in the regions corresponding to the amino acids added, the assay revealed radioactivity in the solvent front.

The record shown was obtained using 0.6-mm Teflon tubing between UV detector and fractionator. Even less loss of resolution was obtained when 0.3-mm tubing was substituted. With a flow-rate of 1 ml/min, we collected fractions of 125 μl every 7.5 s. The proline peak, which the UV detector measured 21 s wide at half-height, was 23 s wide at half-height on the radioactivity record. Despite the finding of radioactivity in the solvent front, the greatest part of the injected radioactivity was recovered in the appropriate fractions.

Examples of applications of radiochromatography

Estimating racemization of pipecolic acid in vivo. Pipecolic acid is a metabolite of the amino acid lysine. In an analysis of the amino acid composition of urine from a patient with hyperlysinemia it was noted that the D-isomer of pipecolic acid was present in excessive quantities along with the L-isomer. The possibility that this unexpected isomer resulted from an *in vivo* racemization reaction was tested by incu-

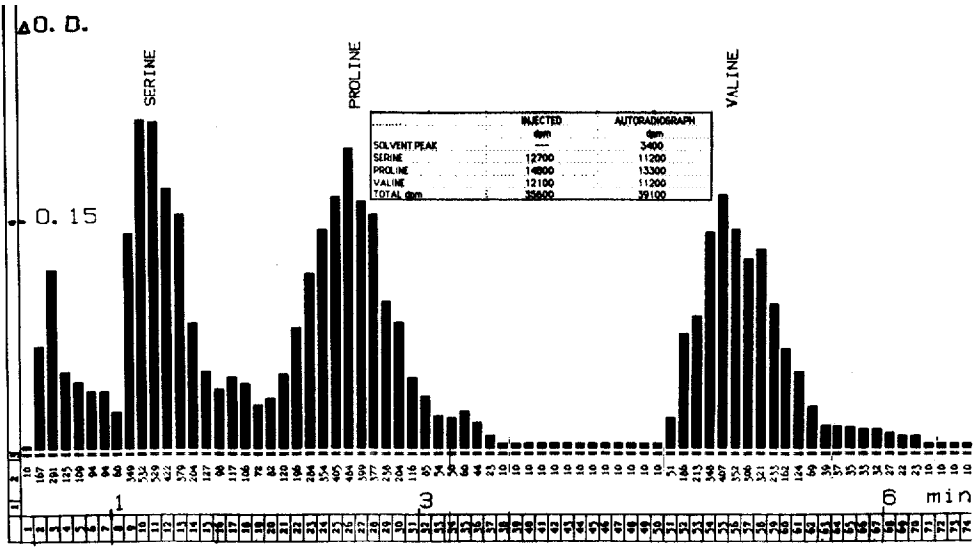

Fig. 1. Assay of radioactivity in a mixture of amino acid standards, containing a total of 35 600 dpm of ^{14}C. The HPLC effluent was fractionated into 80 fractions. Radioautography was carried out for 6 h.

bating ^{14}C-labeled L-isomer with rat liver slices according to the procedure described by Dancis and Hutzler[9]. Incubation was terminated by boiling, and the denatured protein was removed by centrifugation. The supernatant was analyzed by the reversed-phase HPLC method developed in our laboratory[4]. Buffer, containing 295 mg L-aspartame (L-aspartyl-L-phenylalanine methyl ester) and 100 mg of copper sulfate in 1 l of water was pumped through the column overnight prior to the analyses to charge the stationary phase. The mobile phase was 50 ml of the above aspartame–copper sulfate solution, 100 mg of copper sulfate and 0.5 ml glycerine, all diluted to 1 l with water. Pipecolic acid was detected by the UV absorbance of the copper complex at 234 nm. The column effluent was fractionated into 150–200-μl aliquots, evaporated to near-dryness, transferred to chromatography paper and autoradiographed as described.

Both D- and L-pipecolic acid were identified in the specimen by retention times on the record of the UV detector (Fig. 2). The same system separated a known mixture of D- and L-[^{14}C]pipecolic acids (Fig. 3). The liver slices incubated with L-pipecolic acid showed no detectable D-isomer. Only samples that contained up to 300 dpm could be injected into the column seriously overloading it. At this level of radio-

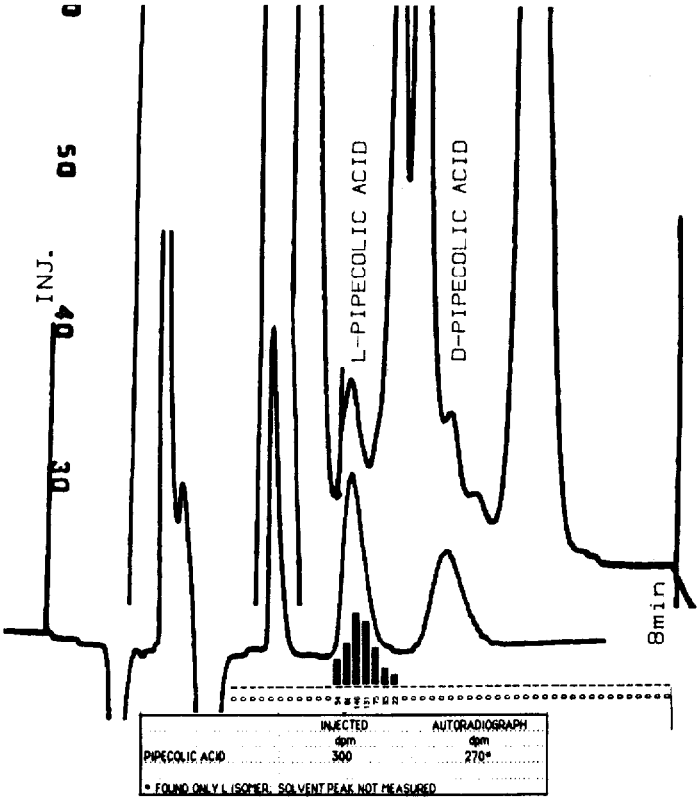

Fig. 2. Chromatogram of pipecolic acid in the supernatant of a rat liver slice preparation for estimating racemization of pipecolic acid *in vivo*. Upper tracing is that of the UV detector, the bar graphs show the radioactivity. A chromatogram of a standard mixture of L- and D-pipecolic acid is superimposed.

activity, racemization would have been detected only if at least 20% of the L-isomer was converted.

Estimation of collagen synthesis in fibrotic liver. The rate of synthesis of collagen in liver slices can be estimated by measuring the rate of conversion of radioactively labeled proline to hydroxyproline. To determine whether the rates differed in normal rat liver and in liver made fibrotic experimentally by infection with the parasite, *Schistosoma mansoni*[5], [^{14}C]proline was incubated with slices of liver from both sets of animals. Slices were incubated with proline diluted to a final specific activity of 1 μCi/μmol. After incubation, the liver proteins were precipitated with chilled ethanol, and the proteins were recovered by centrifugation and dialyzed against distilled water to remove the excess non-protein radioactive amino acid. The remaining protein was lyophilized and subsequently hydrolyzed before analyzing for [^{14}C]proline and [^{14}C]hydroxyproline by the HPLC procedure described above for the analysis of pipecolic acid, followed by fractionation and radioassay.

One set of analyses, in which aliquots containing *ca.* 1250 dpm were injected into the column, showed good recovery of the [^{14}C]proline, but no hydroxyproline. Another set, from an animal with a liver becoming fibrotic, showed identifiable [^{14}C]hydroxyproline as well as the proline (Fig. 4). The hydroxyproline peak contained *ca.* 100 dpm. The hydroproline was not separated from other compounds in the sample. The identification of the radioactive peak, however, is based on the same retention time as the standard hydroxyproline, as shown by the record of the analysis superimposed on that of the test mixture.

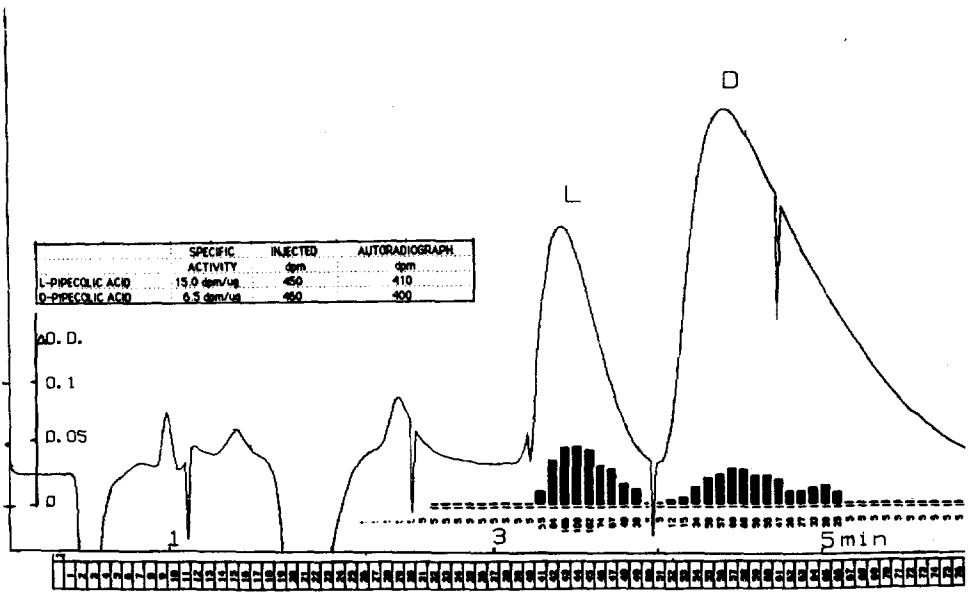

Fig. 3. Assay of radioactivity of a mixture of D- and L-pipecolic acids superimposed on the tracing of the UV detector.

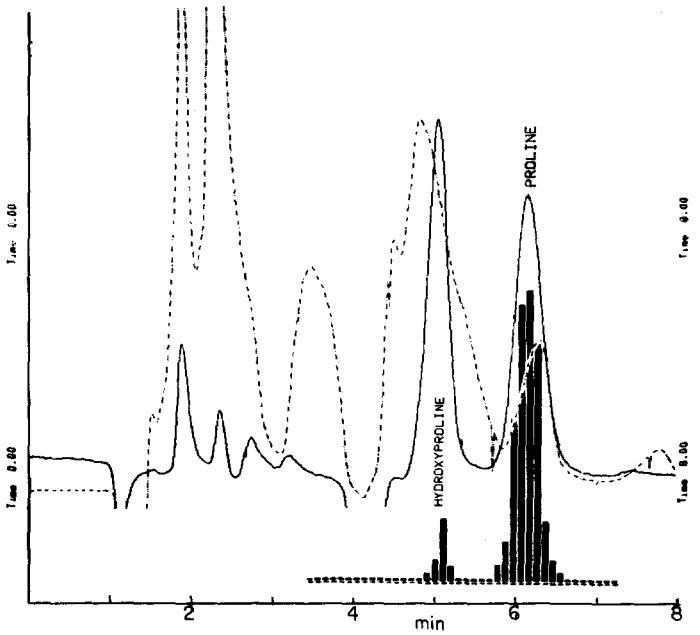

Fig. 4. Chromatogram of hydroxyproline and proline in the supernatant of a liver slice preparation synthesizing collagen as a result of a parasitic infestation. The slices were incubated with radioactive proline. Conversion of radioactive proline to hydroxyproline indicates the synthesis of collagen. The response of the UV detector to the mixture is shown as a dotted line. A chromatogram of hydroxyproline and proline (solid line) is superimposed for reference.

DISCUSSION

Because of the random nature of radioactive emission, the precision of any method of radioassay is a function of the number of radioactive events that contribute to the measurement which, in turn, is a function of the time the radioactive samples remain in the detector. In assays of mixtures separated by packed column gas–liquid chromatography, performed in 30–40 min, samples containing at least 30 000 dpm of ^{14}C can be assayed with efficient flow-through radiation detectors, using flow cells with residence times (volume/flow-rate) of 15–20 s. Detection of individual compounds with 500 dpm or more is possible. With slower chromatographic systems, individual portions of the column effluent can be permitted to remain in the detector for longer assay times without materially degrading resolution and somewhat lower levels of radioactivity can be measured. When samples contain appreciably less radioactivity, 1000 dpm or less, assays generally require fractionation of the effluent and subsequent, off-line radioassay. The time for assaying individual fractions can then be extended as needed for additional precision.

Preserving the certainty of identification of radioactive compounds in off-line radioassay requires that a great many samples be assayed. Since the radioactivity in each fraction is small, long assay times are often required to distinguish radioactivity in the sample from fluctuations in background counting rate. If a single radiation detector is used, the number of samples and the time each must remain in the detector limit the number of samples that can be assayed in any working period.

Many of these limitations are overcome by the approach described here. The HPLC effluent can conveniently be separated into many equal sized fractions by well known techniques. Collecting these fractions on non-wetting fluorocarbon film permits many fractions to be concentrated to near dryness simultaneously and to be transferred to a more mechanically stable paper matrix quantitatively. The small size of the "spot" that results from each fraction is ideally suited for autoradiographic detection in which film blackening is a function of the radioactivity per unit area of paper.

It was at least theoretically simple to devise an adaptation of a well-known fraction collector for use with this system. This fraction collector permitted 80 fractions to be collected in one run. This number is somewhat less than might be desired for radioassay of an entire HPLC separation. Individual aliquots of up to 300 μl can be collected with this system. Using nominal flow-rates of 1 ml/min, and analysis times of 40 min, a fractionator with a capacity of 200 samples or more would be better. The device described here was used to assay parts of an analysis, rather than the whole.

With 72-h film exposure, 10 dpm per spot can be measured: with an exposure time of one week, 5 dpm per spot can be measured. About 50 dpm in a chromatographic peak 1 min wide, with the radioactivity distributed in three fractions, required a one week's exposure. There was minimal enhancement of darkening with fluorescence intensifier screens or with the spray reagents used for tritium detection.

With mechanized collection of fractions, the most labor intensive parttt of this procedure with the equipment described was the scanning of the photographic film. More automated scanning devices are available for scanning TLC plates of similar dimensions that presumably could be used. The use of film strips in a scanner that resembles a motion piction projector is yet another possibility.

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