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# Sensitive and selective liquid chromatographic postcolumn reaction detection system for biotin and biocytin using a homogeneous fluorophore-linked assay

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ABSTRACT

A homogeneous fluorophore-linked assay was used to develop a postcolumn reaction detection system for high-performance liquid chromatography (HPLC). Biotin and biocytin were chosen as the model analytes. The effluent from the HPLC column was merged with a reagent stream containing avidin that was labeled with fluorescein isothiocyanate (avidin-FITC). The binding of the separated analytes by the labeled avidin was accompanied by an enhancement of the fluorescence intensity at 520 nm. This increase in fluorescence was proportional to the concentration of the analytes and constituted the analytical signal. The procedure was optimized with respect to the reagent concentration and the flow-rate of the reagent solution. Analytical characteristics of the method were determined. The procedure was highly selective for biotin and its derivatives. The detection limits for biotin and biocytin were 89 and 94 pg, respectively, for 20-$\mu$l injections. The developed postcolumn reaction detection system was validated by determining biotin in a liquid vitamin preparation and a horse-feed supplement.

## INTRODUCTION

High-performance liquid chromatography (HPLC) has become a valuable tool for the study of complex mixtures. The range of separations available makes it an excellent choice for the characterization of biological samples. At present, UV–Vis absorbance, fluorescence, and electrochemical detectors are the most commonly used HPLC detectors. The applicability of these detectors can be further expanded by using suitable derivatization techniques to convert the analytes into compounds that can be detected with high sensitivity and selectivity. Indeed, coupling chemical reactions to compound-detection following chromatographic separation (*i.e.*, postcolumn) has become an acceptable and widely used means of analysis [1–5].

This article presents a novel postcolumn-reaction detection system based on principles of homogeneous fluorophore-linked assays (a general term that includes homogeneous fluoroimmunoassays). Although the use of these assays in bioanalytical chemistry has been growing because of their specificity and detection limits [6–9], their practical application has been hindered by the many fluorescing compounds, such as proteins and pigments (*e.g.*, bilirubin) that are present in biological samples. Consequently, the coupling of homogeneous fluorophore-linked assays to separation techniques, such as HPLC, should solve the problem of interferences caused by components of the sample matrix, and at the same time, provide highly selective and sensitive detection systems for biologically important compounds.

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In this study, the feasibility of using homogeneous fluorophore-linked assays as a means of reaction detection in HPLC was evaluated by using biotin and biocytin as model analytes. Biotin and biocytin were selected because of their biological significance [10–12]. In addition, the two compounds have very similar properties and, therefore, a separation step is required in their determination [13,14]. Although HPLC seems to be the method of choice for determining biotin and biocytin in complex natural matrices, the absence of strong chromophores or fluorophores in these analytes has precluded their sensitive detection by common HPLC detectors. Consequently, the HPLC methods of separation and determination of these analytes described in the literature [14–16] make use of UV detection in the 205–230 nm range. Attempts have been made to improve the detectability of biotin and its analogues via precolumn derivatization techniques that produce UV-absorbing [17] or fluorescent [17–19] species. Although derivatization has resulted in appreciably improved detection limits, the procedures were time-consuming and did not lend themselves readily to automation.

Recently, two postcolumn reaction detection systems for the HPLC determination of biotin and biocytin based on the competitive-binding principle have been developed [20,21]. One of these systems [20] took advantage of the displacement of the dye 2-(4′-hydroxyphenylazo)benzoic acid (HABA) from its complex with avidin by the analytes, which was monitored by a UV detector at 345 nm. The second system [21] employed a similar displacement reaction using a fluorescent probe, 2-anilinonaphthalene-6-sulfonic acid, and fluorometric detection at 438 nm.

In this article, a postcolumn reaction detection system was designed and evaluated based on a homogeneous fluorophore-linked assay. The system made use of the approximately two-fold enhancement of the fluorescence intensity of fluorescein-labeled avidin upon the binding of biotin or its derivatives [22,23]. Other binding characteristics of the fluorescein-labeled avidin can be found in ref. 22. Because in this case there is no competition between a labeled and an unlabeled ligand for the binding protein, this system is a direct fluorophore-linked assay, rather than the more common competitive binding fluorophore-linked assay. It was anticipated that the application of this assay as a reaction detection system for the HPLC determination of biotin and biocytin should result in further improvement in the detection limits and selectivity of the procedure.

## EXPERIMENTAL

### *Apparatus*

The experimental setup used in this work is shown in Fig. 1. It consisted of a Rainin (Woburn, MA, USA) HPLC system interfaced with a Macintosh Plus computer (Apple Computer, Cupertino, CA, USA). The system included a Rainin Rabbit solvent-delivery system, a Rheodyne Model 7125 injector with a 20-$\mu$l sample loop (Berkeley, CA, USA), and a Knauer Model 87 variable-wavelength UV–Vis detector set at 220 nm. Reversed-phase separations were achieved by using a 5-$\mu$m Microsorb $C_{18}$ column (250 × 4.6 mm I.D.) (Rainin) operated at ambient temperature, which was preceded by a 5-$\mu$m Microsorb $C_{18}$ guard column (15 × 4.6 mm) (Rainin).

The effluent stream from the HPLC column was mixed with the reagent stream containing the avidin labeled with fluorescein isothiocyanate (avidin-FITC). The binding of the analytes to the labeled avidin resulted in an enhancement of the fluorescence intensity. The reagent solution, pumped by an ISCO Model LC-2600 syringe pump (Lincoln, NE, USA), was added to the column effluent through a tee-connector followed by a 10.0-m knitted open-tubular (KOT) reactor made from PTFE tubing (0.5 mm I.D., 14 mm helix diameter) prepared after Krull (KOT2 from ref. 24). The choice of this reactor was based on a previous work [20]. Unless otherwise stated, postcolumn reaction detection was carried out by using a Perkin-Elmer Model LS 50 luminescence spectrometer (Norwalk, CT, USA) with a $\mu$-fluorescence flow cell (20-$\mu$l cell volume, NSG Precision Cells, Farmingdale, NY, USA). For the postcolumn reaction system employing the avidin-FITC reagent, the excitation

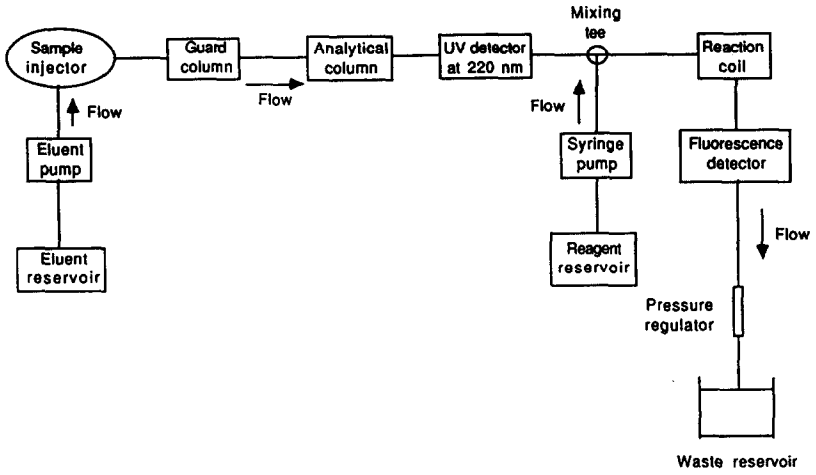


Fig. 1. Schematic diagram of an HPLC postcolumn reaction system for the fluorometric detection of biotin and biocytin employing a homogeneous fluorophore-linked assay.

was at 490 nm and the emission was monitored at 520 nm; the excitation and emission slits were set at 5 and 20 nm, respectively. The fluorescence intensity was recorded in arbitrary units.

Postcolumn reaction detection was also carried out by a Fluorolog-2 spectrofluorometer (SPEX Industries, Edison, NJ, USA) with the same $\mu$-fluorescence flow cell. Fluorescence detection employed the same excitation and emission wavelengths as the previous LS 50 spectrometer. However, the respective slitwidths were set at 2 mm for the SPEX spectrofluorometer. With both fluorometers, a back-pressure regulator was placed at the detector outlet to eliminate outgassing problems.

*Solvent system*

The solvent system for the separation of biotin and biocytin consisted of aqueous 0.100 *M* phosphate buffer, pH 6.0 (solvent A), and 0.200 *M* phosphate buffer (pH 6.0)–methanol (50:50, v/v) (solvent B). Solvents were filtered through a 0.4-$\mu$m membrane filter (Nuclepore, Pleasanton, CA, USA) before use. A 54:46 (solvent A–solvent B) ratio of the two solvents was used to provide baseline resolution of biotin and biocytin in an isocratic mode. A mobile phase flow-rate of 0.40 ml/min was used in all the experiments.

*Reagents*

Biotin, biocytin, monobasic sodium phosphate (reagent grade) and avidin-FITC (with 3.9 fluoresceins attached per avidin molecule) were purchased from Sigma (St. Louis, MO, USA). N,N-Dimethylformamide (DMF) (ACS reagent grade) and acetone (spectrophotometric grade) were obtained from Aldrich (Milwaukee, WI, USA). Methanol (HPLC reagent grade) and methyl ethyl ketone (MEK) (certified) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized (Milli-Q water purification system; Millipore, Bedford, MA, USA) distilled water was used to prepare all solutions.

Biotin and biocytin stock solutions ($4.0 \cdot 10^{-4}$ *M*) were prepared by dissolving the compounds in the mobile phase. Standard solutions were made by further dilutions with the mobile phase. A stock solution of avidin-FITC (50 mg/l, which corresponds to $7.5 \cdot 10^{-7}$ *M*) was prepared in 0.100 *M* phosphate buffer, pH 7.0, and stored refrigerated in an amber bottle. Unless otherwise specified, fresh working solutions of this reagent were prepared daily from the stock solution by dilution with the same buffer.

*Characterization of the avidin-FITC reagent*

A standard $1 \times 1$ cm quartz cuvette was used in all batch mode fluorescence experiments. A

$5 \cdot 10^{-5}$ *M* solution of biotin was added to a cuvette containing 2.0 mg/ml avidin-FITC and the enhancement of fluorescence intensity as a function of reaction time was measured with the LS 50 spectrometer. These data were used to determine the time required for equilibrium to be attained (represented by maximum fluorescence emission intensity).

*Characterization of the postcolumn reaction detection system*

The developed system was optimized in terms of the detection sensitivity by varying the avidin-FITC concentration, the flow-rate of the reagent solution, and the fraction of organic modifier in the reagent solution. Sensitivity, detection limits, linearity of response, precision, and selectivity of the postcolumn reaction system were determined and compared to direct UV detection of biotin and biocytin at 220 nm. A comparison between two different fluorescence detectors (one of them operating in a photon-counting mode) was made. The stability of the avidin-FITC reagent and the cost per analysis of the developed system were also estimated.

*Real sample analyses*

The postcolumn reaction detection system was validated by determining the biotin content of a liquid vitamin preparation and a horse-feed supplement. Specifically, a 50-$\mu$l volume of ABDEC liquid multi-vitamin supplement for infants and children under 4 years of age (Parke-Davis, Morris Plains, NJ, USA), containing 0.05 mg/ml biotin, was diluted to 2.00 ml with the mobile phase. Filtered volumes of 20 $\mu$l of the resulting solution were analyzed by HPLC using the simultaneous direct UV detection at 220 nm and the developed postcolumn reaction system with the fluorometric detection. The two detectors were connected in series, as indicated in Fig. 1. The biotin content in the sample was determined from the calibration curve based on injections of 20-$\mu$l volumes of standard biotin solutions.

In addition, biotin was extracted from two weighed samples (around 0.10 g) of a horse-feed supplement (Gen-a-Hoof from Nickers International, Staten Island, NY, USA) with 10.0 ml of 1.0 *M* NaOH. The pH of 6.00-ml aliquots of these solutions was adjusted to between 6 and 7 with 1.0 *M* HCl. The solutions were further diluted to 100.0 ml with the pH 6.0 phosphate buffer (0.100 *M*), and 20-$\mu$l volumes of the resulting solutions were analyzed after filtration by the procedure described for the liquid vitamin preparation analysis.

## RESULTS AND DISCUSSION

The described postcolumn reaction detection system is based on the natural ability of avidin to bind biotin. When biotin and its analogues associate with avidin labeled with fluorescein, the fluorescence emission signal is enhanced [22].

Isocratic elution with the mobile phase described in the experimental section provided a baseline resolution of biotin and biocytin. A typical chromatogram representing postcolumn reaction detection of 0.10 nmol of the analytes using the avidin-FITC reagent is shown in Fig. 2. This chromatogram indicates that a homogeneous fluorophore-linked assay approach can indeed be used for the sensitive detection of biotin and biocytin.

The dependence of the detector response on

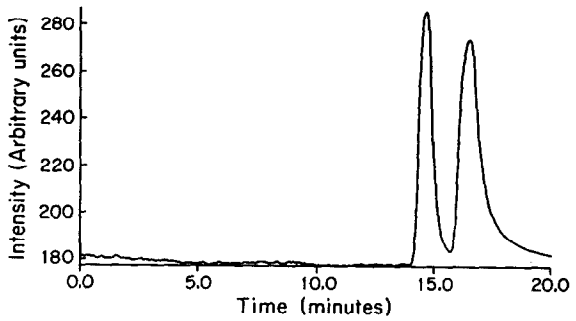


Fig. 2. Typical chromatogram of 20 $\mu$l of a solution containing $5.0 \cdot 10^{-6}$ *M* biotin (first peak) and $5.0 \cdot 10^{-6}$ *M* biocytin (second peak) using the postcolumn reaction system with fluorometric detection. Mobile phase: solvent A–solvent B (54:46); solvent A: 0.100 *M* phosphate buffer, pH 6.0; solvent B: 0.100 *M* phosphate buffer, pH 6.0, in water–methanol (50:50, v/v). Mobile phase flow-rate: 0.40 ml/min. Postcolumn reagent: 2.0 mg/l avidin-FITC in 0.100 *M* phosphate buffer, pH 7.0. Reagent flow-rate: 1.00 ml/min. A 10.0-m KOT reactor was used for postcolumn reaction detection. The remaining conditions are described in the Experimental section.

the concentration of the avidin-FITC was studied at three concentration levels of the reagent solution: 1.0, 2.0 and 3.0 mg/l avidin-FITC. In this experiment, the flow-rate of the reagent solution was 1.00 ml/min and the concentration of the analytes was kept constant at $5.0 \cdot 10^{-6}$ *M*. It was found that an increase in the reagent concentration from 1.0 to 2.0 mg/l avidin-FITC resulted in an enhancement of the response of the fluorometric detector by 80 and 64% for biotin and biocytin, respectively. In contrast, a further increase in the reagent concentration from 2.0 to 3.0 mg/l avidin-FITC caused only a small enhancement of the detector response, not exceeding 20%. On the basis of these data, a reagent concentration of 2.0 mg/l avidin-FITC was selected for further experiments, this value being a compromise between detector response and reagent consumption (*i.e.*, cost of analysis).

The effect of the flow-rate of the reagent solution on the detector response was examined by varying the reagent flow-rate in the 0.40–1.20 ml/min range, while keeping constant the amount of biotin and biocytin injected (0.10 nmol), the mobile phase flow-rate (0.40 ml/min), as well as the other chromatographic conditions. This dependence is shown in Fig. 3. The presence of a maximum can be accounted for as follows: an initial increase of the detector response with the flow-rate is consistent with the law of mass action, *i.e.*, the supply of the reagent available for the binding of the analytes increases

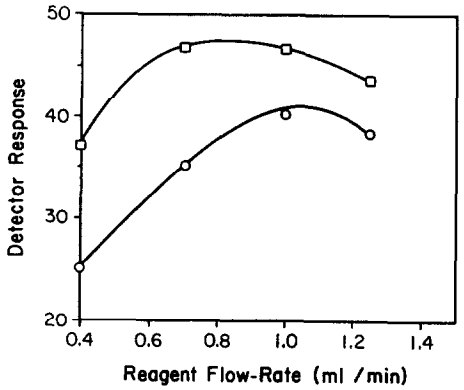


Fig. 3. Dependence of the detector response (expressed as peak height) for 0.10 nmol of biotin (□) and biocytin (○) on the reagent flow-rate. Reagent concentration: 2.0 mg/l avidin-FITC. For the remaining conditions, see Fig. 2.

with the flow-rate. A decrease in the detector response at high flow-rates (over 1.00 ml/min) is presumably due to the dilution of the column effluent stream, which in turn lowers the analyte concentration at the detector, and to insufficient reaction time. Indeed, batch mode experiments (measuring the change in fluorescence intensity as a function of time upon addition of biotin/biocytin to avidin-FITC) indicated that binding reactions were complete within 3 min. However, at higher flow-rates the reaction time in the postcolumn reaction detection system was in the order of 1 min. To maximize the detector response, the flow-rate of the reagent used throughout the remaining experiments was kept at 1.00 ml/min. The cost of the reagent per analysis estimated for these chromatographic conditions was under US$ 0.50.

The type and content of organic modifier are known to influence the fluorescence properties of fluorophores. Therefore, the effect of the methanol content on the detector response was examined by separating 0.10 nmol of biotin and biocytin using the optimum conditions for postcolumn reaction detection, but the reagent solution in this experiment was prepared in 0.100 *M* phosphate, pH 7.0 buffer–methanol (50:50, v/v). The resulting chromatograms exhibited a lower fluorescence background compared to the ones obtained by using the aqueous reagent solution (the remaining chromatographic conditions were the same), and no detector response for the analytes was observed. This effect was presumably due to denaturation of avidin by methanol and/or to fluorescence quenching. Consequently, the reagent solution used in the remaining studies was aqueous.

The repeatability of the results was examined by determining the relative standard deviation (R.S.D.) of the detector response that corresponds to 20-$\mu$l injections of $5.0 \cdot 10^{-6}$ *M* biotin and biocytin. In the case of retention times, the R.S.D. was 0.25%, whereas for the peak heights it ranged from 2.8 to 3.1% ($n = 8$). These results indicate that the developed procedure has the typical precision of chromatographic determinations.

The developed reaction detection system was evaluated in terms of its selectivity properties by

analyzing a series of solutions containing 0.10 nmol of the analytes and increasing amounts of three other organic compounds: DMF, acetone and MEK. Under the chromatographic conditions used in the present study, these compounds have similar retention times as biotin and biocytin, and their molecules contain chromophores absorbing at 220 nm. It was established that when the proposed reaction detection scheme was used, concentrations of DMF, acetone and MEK as high as $7 \cdot 10^{-2}$ $M$ did not affect the detector response. In contrast, the direct absorptiometric detection at 220 nm suffered from severe interference by these compounds due to peak overlapping, which precluded correct determination of either of the two analytes. Therefore, it may be concluded that the selectivity of the developed procedure is primarily controlled by the binding characteristics of the binding protein employed (avidin).

The developed procedure was further characterized by constructing calibration curves for biotin and biocytin that were based on the chromatographic peak heights and peak areas. The data for these curves were obtained under the optimum conditions for the reaction detection. For the LS 50 detector, the curves for the two analytes were linear from $5 \cdot 10^{-7}$ to $1 \cdot 10^{-5}$ $M$ (*i.e.*, 0.01 to 0.2 nmol for a 20-$\mu$l sampling loop). An increase in the concentration of the analytes over $1.0 \cdot 10^{-5}$ $M$ was accompanied by an appreciable peak broadening and deteriorated resolution, and above $2.0 \cdot 10^{-5}$ $M$ the calibration curves leveled off. A photon-counting spectrofluorometer was also evaluated as a detector in this system. In this case, the calibration curves were linear from $4 \cdot 10^{-8}$ $M$ to $6 \cdot 10^{-7}$ $M$. In addition, it was determined that the introduction of the KOT reactor did not result in appreciable band broadening. Typically, the peak width increase due to the KOT reactor was about 15%. Further, the resolution of the postcolumn HPLC system toward biotin and biocytin did not change significantly.

The detection limits were estimated by analyzing a series of biotin and biocytin solutions of decreasing concentrations, until a signal-to-noise ratio ($S/N$) of 3 was obtained. Using the LS 50, the detection limits for both analytes were found to be $1.4 \cdot 10^{-7}$ $M$, which corresponds to 0.66 ng biotin and 1.0 ng biocytin for a 20-$\mu$l sample volume. The detection limits when using the photon-counting detector were estimated to be 89 and 94 pg for biotin and biocytin, respectively. These values were almost an order of magnitude lower than those obtained from the LS 50. The better detection limits were attributed to the photon-counting mode of detection. This constitutes an improvement with respect to the UV detection at 220 nm by a factor of 67 and $2.6 \cdot 10^{2}$ for biotin and biocytin, respectively.

The analytical utility and the accuracy of the proposed procedure were examined by determining the biotin content in a commercial liquid vitamin preparation and in a horse-feed supplement. The amount of biotin found in the liquid vitamin supplement ABDEC was 0.051 mg/ml (average from two determinations), which is in good agreement with the amount claimed by the manufacturer (0.05 mg/ml). The composition of 1 ml of ABDEC is: 1500 international units (I.U.) of vitamin A, 400 I.U. of vitamin D, 5 I.U. of vitamin E, 35 mg of vitamin C, 0.5 mg of thiamine, 0.6 mg of riboflavin, 8 mg of niacin, 0.4 mg of vitamin $B_6$, 2 $\mu$g of vitamin $B_{12}$, 0.05 mg of biotin, and 3 mg of pantothenic acid.

The selectivity of the postcolumn reaction detection system for biotin over the other ten vitamins present in the ABDEC preparation was excellent. This can be perceived by comparing two chromatograms of the vitamin solution obtained with the direct UV detection at 220 nm (Fig. 4A) and by employing the postcolumn reaction system with the fluorometric detection (Fig. 4B). It is evident that the interference from the other sample components precludes a correct quantitative determination of biotin when using the direct detection at 220 nm, whereas no interference is observed for the developed postcolumn reaction detection system. The difference in retention times for biotin between the chromatograms in Fig. 4A and B is a result of the additional time required for the eluent to pass through the 10.0-m KOT reactor.

Likewise, a horse-feed supplement was analyzed that contained, in addition to biotin (231 mg/kg), zinc methionine, maltodextrin, and ground rice hulls. Two samples of the supple-

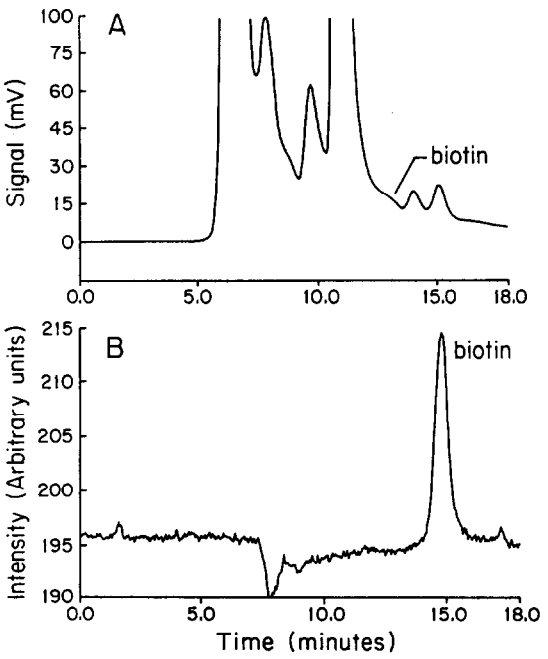


Fig. 4. Chromatograms of 20 $\mu$l of a diluted solution (1:40, v/v) of the liquid vitamin preparation ABDEC: (A) absorbance detection at 220 nm, (B) postcolumn reaction with fluorometric detection. For the chromatographic conditions, see Fig. 2.

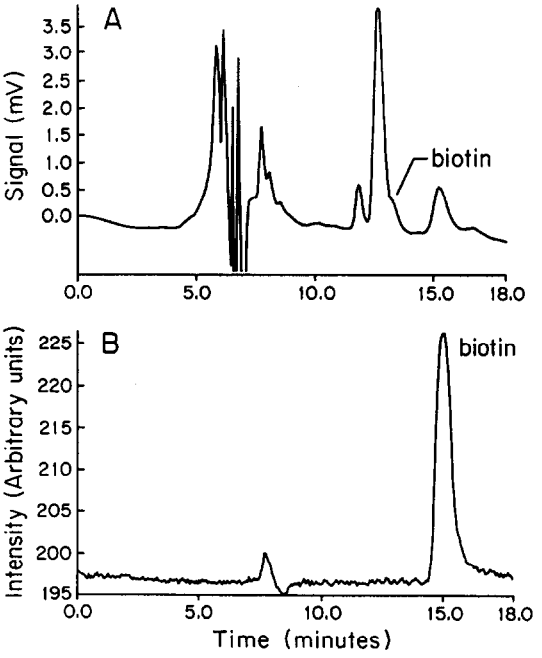


Fig. 5. Chromatograms of 20 $\mu$l of the extract of a horse-feed supplement: (A) direct UV detection at 220 nm, (B) postcolumn reaction with fluorometric detection. For the chromatographic conditions, see Fig. 2.

ment were extracted and analyzed for the biotin content by following the procedure outlined in the experimental section. As shown in Fig. 5, the UV detection at 220 nm suffered from severe interferences, while the chromatogram obtained by using the developed reaction system revealed the presence of only the biotin peak. The quantitative determination of biotin in the two samples yielded the following results: 240 and 236 mg/kg. The average value, 238 mg/kg, is in good agreement with the biotin content claimed by the manufacturer (the difference amounting to 3%).

The stability of the postcolumn reagent was evaluated by filling the syringe pump with 2.0 mg/l avidin-FITC and using it repetitively to analyze a test solution containing fixed amounts of biotin and biocytin. It was found that the reagent solution was sufficiently stable for at least eight hours as revealed by the unchanged peak heights of biotin and biocytin. However, after 24 h no chromatographic peaks corresponding to the analytes were observed. Apparently, this is due to adsorption of avidin-FITC (present at very low concentration of $3 \cdot 10^{-8}$ $M$) on the walls of the syringe pump. In contrast, the stock avidin-FITC solution was stable for at least two weeks when stored refrigerated in amber vials. As a result, fresh working solutions of avidin-FITC were prepared daily from the stock solution.

## CONCLUSIONS

This study has demonstrated the feasibility of a postcolumn reaction detection system for biotin and biocytin based on a homogeneous fluorophore-linked assay. The developed procedure has an improved selectivity over the direct UV detection at 220 nm. The detection limits were 89 and 94 pg for biotin and biocytin, respectively. The high sensitivity of the described system is a result of the high quantum efficiency of fluorescein. Further, because fluorometric detection is performed at a wavelength in the visible region, the majority of compounds present in natural samples do not interfere. Indeed

the developed method was used to determine the biotin content in real samples with minimum pretreatment. The high selectivity of the described system is a combination of the binding selectivity of avidin (*i.e.*, ability to discriminate between compounds that contain the biotin moiety and those that do not) and the separation process itself (to separate biotin and its analogues).

The applicability of the proposed system is more general and can be extended to postcolumn reaction detection of other analytes as long as there exist fluorophore-labeled biological binders (*e.g.*, antibodies, binding proteins, receptors, and lectins) that undergo spectral changes as a function of the concentration of the analytes. Work in this direction is currently underway in our laboratory.

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