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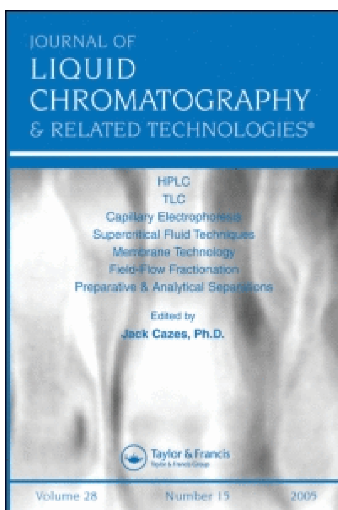

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Discrimination of Primary Amines from Other Fluorescent Compounds in Biological Fluids

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DISCRIMINATION OF PRIMARY AMINES FROM OTHER FLUORESCENT COMPOUNDS IN BIOLOGICAL FLUIDS

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ABSTRACT

Experimental condition and parameters involved in HPLC distinction of primary amines from non-primary amine compounds with native fluorescence were investigated. The discrimination conditions were designed to enable utilization of the sensitive reagent fluorescamine as a means of monitoring primary amines in physiologic fluids.

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INTRODUCTION

Fluorescamine is a fluorogenic reagent which is used for the detection of primary amines at the picomole level (1, 2). This reagent has been incorporated into an amino acid analyzer, as well as into a monitoring system for peptides and proteins in column effluents (3). The monitoring system has been employed in a number of projects involving the isolation of peptides and proteins from tissue extracts. These include opioid peptides (4, 5), interferons (6, 7), growth factors (8) and leukotriene (9), as well as synthetic peptides (10).

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With the anticipated increase in the popularity of this technique, it is important to point out any unusual situations which might arise. During the purification of a biologically active primary amine substance from urine, the presence of a non-primary amine compound with native fluorescence was observed. This situation and the method used to discriminate between the two is reported.

MATERIAL

Aliquots of lyophilized urine from uremic patients and "post salt" fraction, which was isolated by chromatography on Sephadex G-25 followed by HPLC, were prepared as previously described (11, 12).

Pyridine, acetic acid, acetone and methanol were distilled twice. Water was purified by a system obtained from Hydro Service and Supplies (Durham, N.C.). Fluorescamine and 25% solution of thiodiglycol were obtained from Pierce (Rockford, Ill.). All other chemicals used in the preparation of reagents or buffers were from Fisher (Springfield, N.J.).

A prepacked 25 x 0.46 cm column of Lichrosorb RP-18 was obtained from Altex (Berkeley, Ca.). A 25 x 0.46 cm column prepacked with H-70 (cation exchange) was obtained from Hamilton (Reno, Nevada). A model 420 microprocessor and a model 110 A pump were obtained from Altex (Berkeley, Ca.); a Milton-Roy (Riviera Beach, Fla.) minipump and high pressure sample injection valve, Rheodyne (Berkeley, Ca.) were used for the automatic monitoring system (3).

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METHOD

A Lichrosorb RP-18 column was pumped at 1 ml/hr at room temperature with back pressure of 750 psi. A 0.2 M pyridine-acetate buffer pH 5.5 was used for elution. An 80-minute linear gradient from 20% to 50% methanol was used. All column buffers contained thiodiglycol (0.01% v/v), an antioxidant, and caprylic acid (0.01% v/v) to prevent growth of microorganisms. A cation exchange H-70 column was pumped at 11 ml/hr at room temperature and back pressure of 300 psi. Samples were applied to the column in a 200 μl of 0.5 M pyridine-acetate buffer pH 5.5 and eluted with the same buffer.

For the discrimination of natural fluorescence from fluorescamine dependent substances, equal aliquots of the unknown mixture were applied sequentially to the same column. In the first application, an aliquot of the column effluent is transferred into the detector stream where it mixes with the buffer and acetone only. In the second application, an equal aliquot of the column effluent is transferred into the detector stream where it mixes with buffer and fluorescamine. The emitted fluorescence in the first chromatographic pattern reflects only compounds with natural fluorescence; whereas in the second application in the presence of fluorescamine, natural and fluorescamine dependent fluorescent compounds were detected together. Thus, primary amine compounds which are fluorescamine dependent and do not exhibit native fluorescence were discerned from non-primary amine compounds with natural fluorescence.

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RESULTS AND DISCUSSION

Human and dog urine were used in the course of a study directed toward purification and characterization of natriuretic hormone (11-14). In Figure 1, a characteristic pattern is shown when urine from uremic patients was subjected to Lichrosorb RP-18 column. The presence of multiple peaks was shown to be composed of many fluorescamine dependent compounds.

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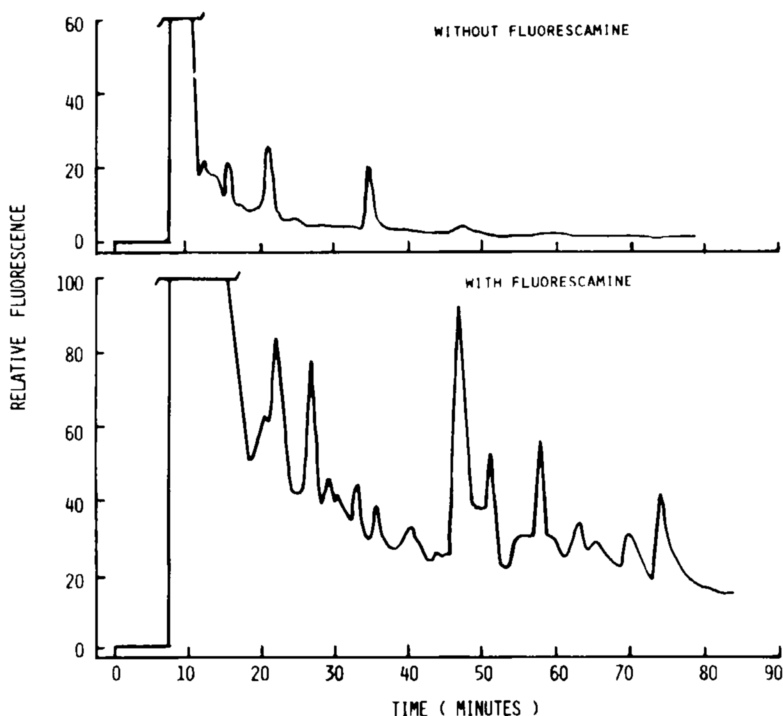

FIGURE 1. Chromatography of uremic patient urine: 200 µl of urine was applied to a 0.46 x 25 cm column of Lichrosorb RP-18 at 25° C. An 80 minutes gradient from 20% methanol and 0.2 M pyridine-acetate pH 5.5 to 50% methanol in the same buffer, was used at a flow rate of 1 ml/min. A portion (2%) of the column effluent was directed to the monitoring system.

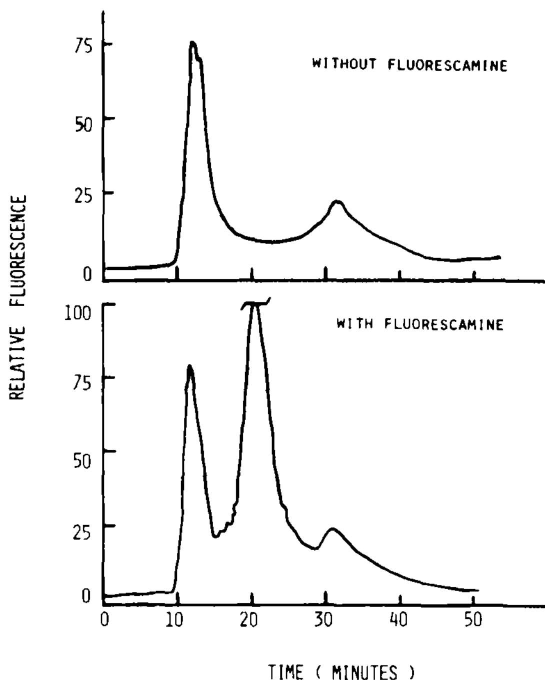

FIGURE 2. Monitoring of "post salt" G-25 Sephadex dog urine: Chromatography of "post salt" G-25 Sephadex fraction on a 0.46 x 25 cm column of H-70 at room temperature. 0.5 M pyridine-acetate pH 3.5 was used for elution, at a flow rate of 11 ml/hr.

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Figure 2 demonstrates the benefit of using the monitoring system when an aliquot of column effluent is transferred to the detector stream where fluorescamine was added or omitted. Three fluorescent peaks were noted, but only one was fluorescamine dependent and did not exhibit spontaneous fluorescence.

The method presented in this paper clearly demonstrates the following advantages: Using the highly sensitive monitoring system, adding or omitting fluorescamine from the detector stream, enabled screening of primary amines in the picomole range. The

high resolving power of reverse phase HPLC permitted the separation of highly complicated primary amine mixtures in relatively short time and with good reproducibility. The recoveries depended on the primary amine but were usually in the 80% range. There was no change in the elution position of standard marker peptides and the sodium transport inhibitor fraction over several months.

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