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USE OF UV SCANNING TECHNIQUES IN THE IDENTIFICATION OF SERUM CONSTITUENTS SEPARATED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Serum constituents separated by the reversed-phase partition mode of high-performance liquid chromatography were identified by using a stopped-flow UV scanning technique. This method of peak identification eliminates post-chromatographic sample handling. Results from UV scanning were correlated with those obtained from absorbance ratios of reference compounds and the enzymatic peak-shift technique.

INTRODUCTION

Although high-performance liquid chromatography (HPLC) can now provide excellent separations of complex mixtures, the elucidation of the chemical nature of individual peaks still presents some problems. This is particularly true with samples of biological origin, which are often available in very small amounts or which contain exceedingly low amounts of the compounds of interest. Although compounds can be tentatively identified by retention times or volumes or co-chromatography with reference compounds, it is important that the results be confirmed by other methods.

Many of the identification techniques commonly used, such as infrared and nuclear magnetic resonance spectroscopy, require large sample sizes. In other techniques, such as mass spectrometry, the samples must be volatile. Thus, the low volatility of certain classes of compounds limits the use of a mass spectrometer as a detector or for identification purposes. The enzymatic peak-shift technique, which utilizes the specificity of an enzyme in catalyzing a reaction, has proved to be a very useful post-chromatographic method of peak identification for certain classes of compounds[1–6]. Its use is limited, however, to those enzymes which are commercially available or to reactions in which the reactants and/or products of the enzymatic reaction absorb in the UV region. With the development of variable-wavelength UV detectors for HPLC, it is now possible to characterize compounds by absorbance ratios[4–7]. If the wavelengths are well chosen, absorbance ratios can provide a very

useful means of peak identification and for the determination of the purity of a compound.

Another method for peak identification is the stopped-flow UV scanning technique, which permits the examination of the entire UV spectrum of the compound under study[7–13]. It is performed on-line with the chromatographic separation and thus eliminates the errors of post-chromatographic sample handling[14]. Although UV spectra are not generally used alone for structural elucidation because of the lack of fine structure in the broad bands, they can be used in combination with other evidence for the positive identification of chromatographic peaks. Therefore, the usefulness of this detector for the identification of peaks was evaluated in the analysis of some naturally occurring serum constituents.

EXPERIMENTAL

Instrumentation

A Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 A solvent delivery system, a Model 660 solvent programmer and a Model U6K universal injector were used in all determinations. An SF 770 Spectroflow monitor with a deuterium lamp, SFA 339 wavelength drive and MM 700 memory module (Schoeffel Instrument Corp., Westwood, N.J., U.S.A.) were used to obtain absorption spectra.

Materials

1,1,2-Trichlorotrifluoroethane (Freon) was purchased from Matheson Gas Products (East Rutherford, N.J., U.S.A.), tri-*n*-octylamine (amine) from ICN Pharmaceuticals (Plainview, N.Y., U.S.A.) and trichloroacetic acid (TCA) and tris-hydroxymethylaminomethane (Trizma base), the enzymes xanthine oxidase (E.C. 1.2.3.2), uricase (E.C. 1.7.3.3), purine nucleoside phosphorylase (E.C. 2.4.2.1), and the reference compounds uric acid (UA), hypoxanthine (HYP), xanthine (XAN), inosine (INO), guanosine (GUO), *l*-tryptophan (L-Trp), 1,3-dimethylxanthine (1,3-Me_2XAN), 3,7-dimethylxanthine (3,7-Me_2XAN) and caffeine were purchased from Sigma (St. Louis, Mo., U.S.A.). Solutions of the reference compounds were prepared at a concentration of 1 mM in 0.01 F KH_2PO_4 (pH 6.0) and kept frozen when not in use. Reagent-grade potassium dihydrogen orthophosphate (KH_2PO_4) was purchased from Mallinckrodt (St. Louis, Mo., U.S.A.).

Chromatographic conditions

The low-concentration eluent was 0.02 F KH_2PO_4 solution, its pH being adjusted to 5.5 using dilute potassium hydroxide solution. Methanol–water (3:2, v/v) was used as a high-concentration eluent. Methanol, distilled in glass, was obtained from Burdic & Jackson (Muskegon, Mich., U.S.A.). Solvents were filtered through Millipore membrane filters, Type HA, pore size 0.45 μm (Millipore, Bedford, Mass., U.S.A.), and degassed under vacuum before use. A 35-min linear gradient from 0 to 40% of the high-concentration eluent was used in all determinations. The flow-rate was 1.5 ml/min and the temperature was ambient.

UV scanning technique

With a deuterium lamp, a wavelength range of 190–500 nm can be scanned

at a rate of 100 nm/min. The spectrum obtained at high sensitivities from dilute samples is distorted by a spectral background due to the changing optical properties of flow cells and monochromator. This background can be stored over the entire wavelength range by the memory module and later subtracted from the sample scan to provide a corrected spectrum. Peak heights at 254 and 280 nm were recorded simultaneously on a two-pen recorder.

Sample preparation

To obtain serum samples, freshly drawn blood was collected in a tube without anticoagulant. The blood was allowed to clot spontaneously for 10–15 min at room temperature. The supernatant fluid was then spun down at 630 *g* for 10 min to remove any contaminating white cells. The liquid was withdrawn and filtered using a Millipore 13-mm stirred ultrafiltration cell with the PTGC Pellicon-type membrane (nominal molecular weight 10,000)[15]. The filtrate was transferred into a plastic vial and kept frozen until the analysis.

For the determination of the total tryptophan content of serum, the sample was deproteinized with cold, 6% (w/w) TCA. To each milliliter of serum, 2 ml of cold TCA were added and the solution was centrifuged for 3 min at 630 *g* to remove the protein. The supernatant fluid was filtered through a plastic Millipore filter (Swinnex-25) and the filtrate transferred into another test-tube to which 1 ml of 0.5 *F* amine–Freon was added for every milliliter of the filtrate. The mixture was vortexed for 3 min at 50 *g*. The top layer was withdrawn and transferred into a plastic vial[16].

Peak identification

Absorbance ratios were calculated by measuring the peak heights at 254 and 280 nm. These values were then compared with the ratios of the unknown peaks in the chromatograms.

For the enzymatic peak shift, an aliquot of the serum sample was incubated with the appropriate enzyme under the specified conditions of pH and temperature. The sample was chromatographed and the chromatogram compared with that of the unreacted serum.

In using the UV scanning technique, a blank gradient was run and the flow stopped at the point at which the compound of interest was eluted. The spectrum was then scanned from 210 to 500 nm and stored in the memory unit. Next, standard solutions of reference compounds were chromatographed under the same conditions, the flow was stopped at the top of each peak and the spectrum scanned. It should be noted that owing to the low solute diffusivity in the mobile phase, diffusion effects are not significant even if the mobile phase flow is arrested for several hours.

RESULTS AND DISCUSSION

The use of the identification techniques is illustrated in the analysis of some serum constituents. First, a tentative identification was made, based on retention times and comparison with the reference compounds. The UV spectra were then obtained by stopped-flow scanning. To standardize the procedure, some reference compounds under study were separated and detected at 254 nm (Fig. 1). Fig. 2 illustrates the uncorrected and the corrected spectra for the blank run. Next, the mixture of reference

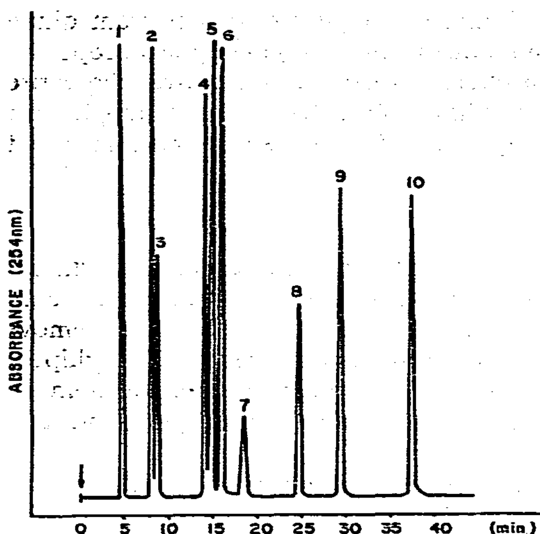


Fig. 1. Separation of some reference compounds. Column: μBondapak/C_{18}. Eluents: low concentration, 0.02 *F* KH_2PO_4, pH 5.5; high concentration, CH_3OH–H_2O (3:2); gradient, linear from 0 to 40% of the high-concentration eluent in 35 min; flow-rate, 1.5 ml/min. Detector: UV at 254 nm. Temperature: ambient. Peaks: 1 = uric acid; 2 = hypoxanthine; 3 = xanthine; 4 = xanthosine; 5 = inosine; 6 = guanosine; 7 = tryptophan; 8 = theobromine; 9 = theophylline; 10 = caffeine.

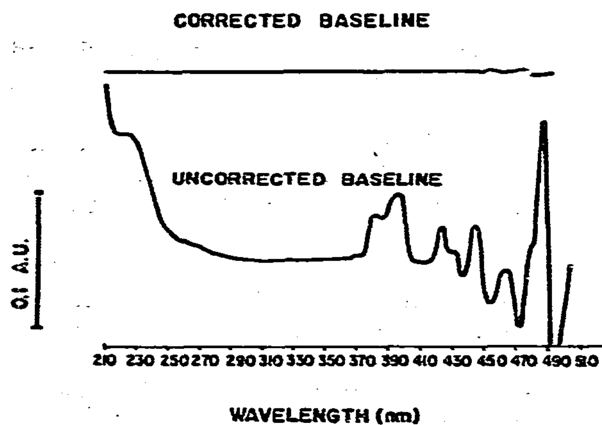


Fig. 2. Corrected *versus* uncorrected background spectrum. Scanning range, from 210 nm to 500 nm; scanning rate, 100 nm/min; absorbance, 0.4 a.u.f.s.

compounds was chromatographed, the flow stopped at the top of the peak under study and the spectrum scanned from 210 to 500 nm. The UV spectra of the components of the mixture of reference compounds are shown in Fig. 3. With biological samples, the spectrum of each compound of interest was then correlated with absorbance ratios, which were calculated at two wavelengths and compared with those of reference compounds. In addition, whenever possible, an aliquot of the sample was incubated with an enzyme to characterize the peaks further.

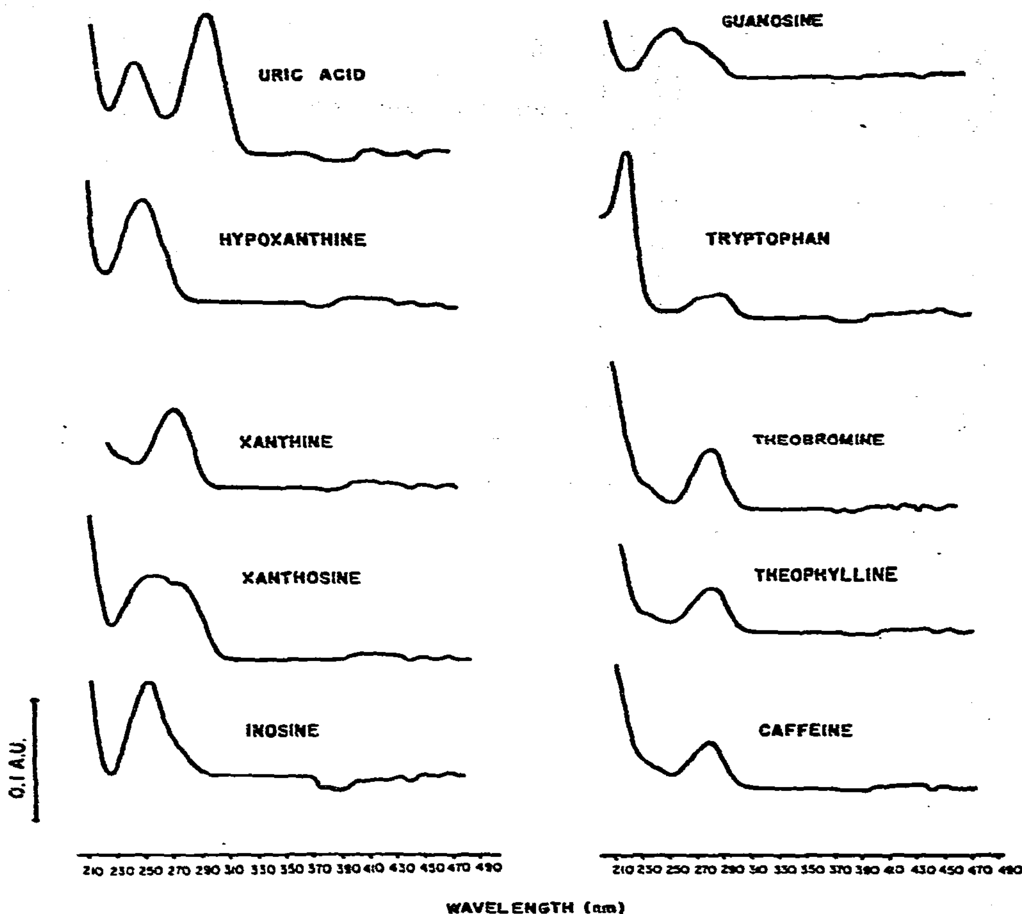


Fig. 3. UV spectra of some reference compounds obtained by stopped-flow scanning technique. Chromatographic conditions as in Fig. 1; scanning conditions as in Fig. 2.

For serum studies, samples of pooled serum obtained from normal subjects and from patients with certain disease states were deproteinated by ultrafiltration. An aliquot of each sample was then chromatographed under the described chromatographic conditions. Fig. 4A illustrates the separation of the constituents of a pooled serum sample. In order to determine the identity of peak 1, which had the retention time of hypoxanthine, the sample was first co-chromatographed with a hypoxanthine standard; Fig. 4B shows the chromatogram of the resulting mixture. An increase in the area of peak 1 indicated that the compound might be hypoxanthine. Next, the UV spectrum of a standard solution of hypoxanthine and peak 1 was scanned; there was close agreement between the two spectra (Fig. 5). The absorbance ratios (280/254 nm) were then determined for the hypoxanthine standard and for peak 1 in the serum, average values of triplicate runs being 0.48 ± 0.02 and 0.49 ± 0.01, respectively; there is good agreement between the ratios of the unknown peak and the reference compound. The enzymatic peak-shift technique with xanthine oxidase

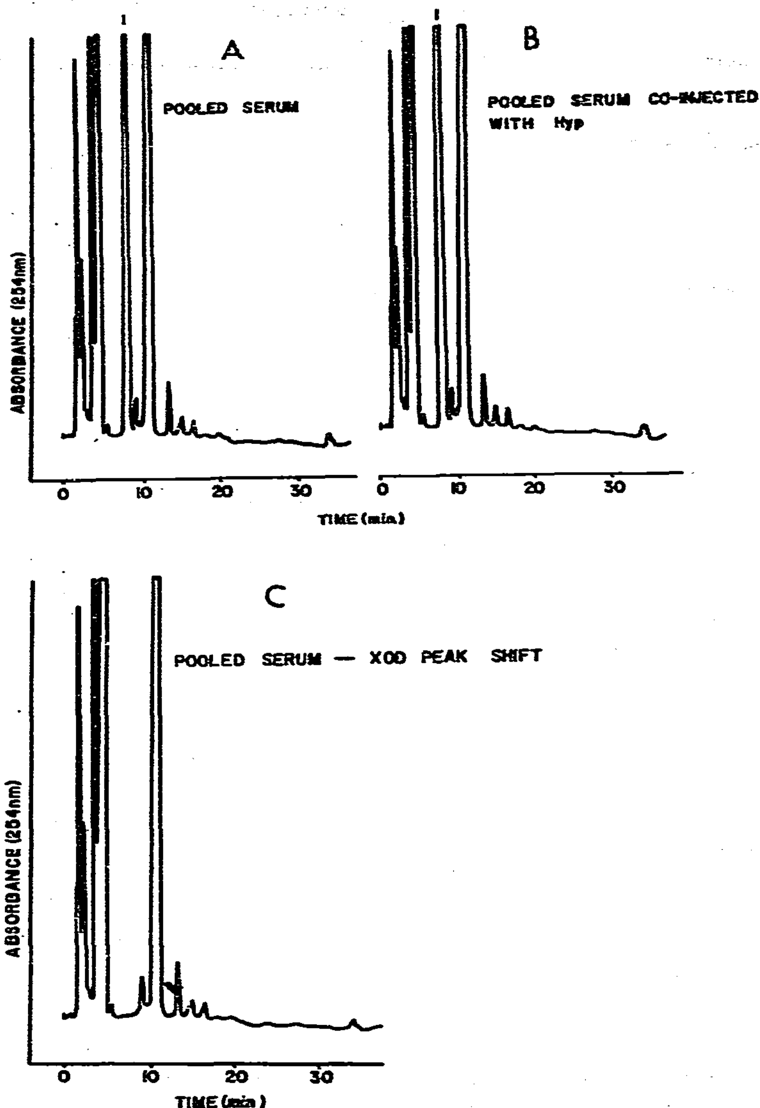
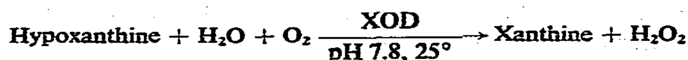
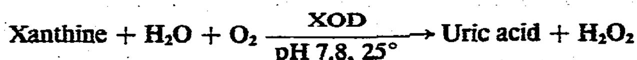


Fig. 4. (A) Chromatogram of a pooled serum sample. (B) Chromatogram of a pooled serum sample co-injected with hypoxanthine. (C) Xanthine oxidase peak shift carried out on a pooled serum sample. Chromatographic conditions for all three chromatograms as in Fig. 1.

(XOD) was used to obtain further proof of the identity of peak 1. The reaction and the conditions under which it was carried out were as follows:



$$\text{Hypoxanthine} + H_2O + O_2 \xrightarrow[\text{pH 7.8, 25}^\circ]{\text{XOD}} \text{Xanthine} + H_2O_2$$



$$\text{Xanthine} + H_2O + O_2 \xrightarrow[\text{pH 7.8, 25}^\circ]{\text{XOD}} \text{Uric acid} + H_2O_2$$

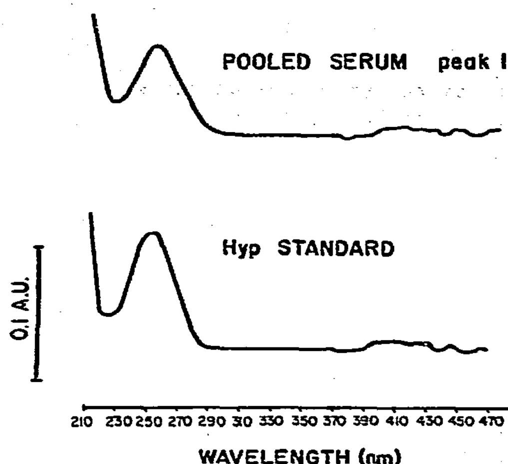
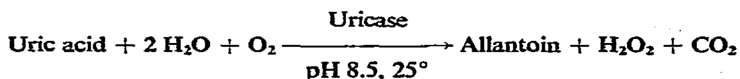


Fig. 5. UV spectra of peak 1 in pooled serum and the hypoxanthine standard. Scanning conditions as in Fig. 2.

The chromatogram of the incubated sample is shown in Fig. 4C. As xanthine oxidase catalyzes the conversion of hypoxanthine and xanthine into uric acid, the disappearance of peak 1 and the simultaneous appearance of a product with the retention time of uric acid was taken as an indication that peak 1 is either hypoxanthine or xanthine. Therefore, from the combined evidence, it is concluded that the peak is hypoxanthine and the peak represents the pure compound.

A chromatogram of a serum sample from a normal subject is shown in Fig. 6A. As peak 1 had the retention time of uric acid, the serum sample was co-chromatographed with the uric acid standard (Fig. 6B). The comparison of the UV spectra of the peak under study and the uric acid standard is shown in Fig. 7. The absorbance ratios (280/254 nm) for peak 1 in normal serum and the uric acid standard (average values of triplicate runs) were 3.53 ± 0.02 and 3.59 ± 0.01, respectively. The close agreement of the ratios indicates that the peak is pure. An aliquot of the sample was then incubated with uricase under the following conditions:



$$\text{Uric acid} + 2\,H_2O + O_2 \xrightarrow[\text{pH 8.5, 25°}]{\text{Uricase}} \text{Allantoin} + H_2O_2 + CO_2$$

In the chromatogram of the incubated mixture (Fig. 6C), it can be seen that peak 1 was removed. The product of the enzymatic reaction, allantoin, does not absorb at 254 nm. Based on the agreement between the UV spectra and accumulated evidence from other identification methods, it was concluded that peak 1 in the serum from a normal subject is uric acid.

As peak 2 in the same sample had the retention time of caffeine, the peak was tentatively identified by co-injection of a standard of caffeine with the sample. The UV spectrum of the peak was then compared to that of caffeine (Fig. 7) and it is clearly indicated that the peak of interest is caffeine. As additional evidence, the

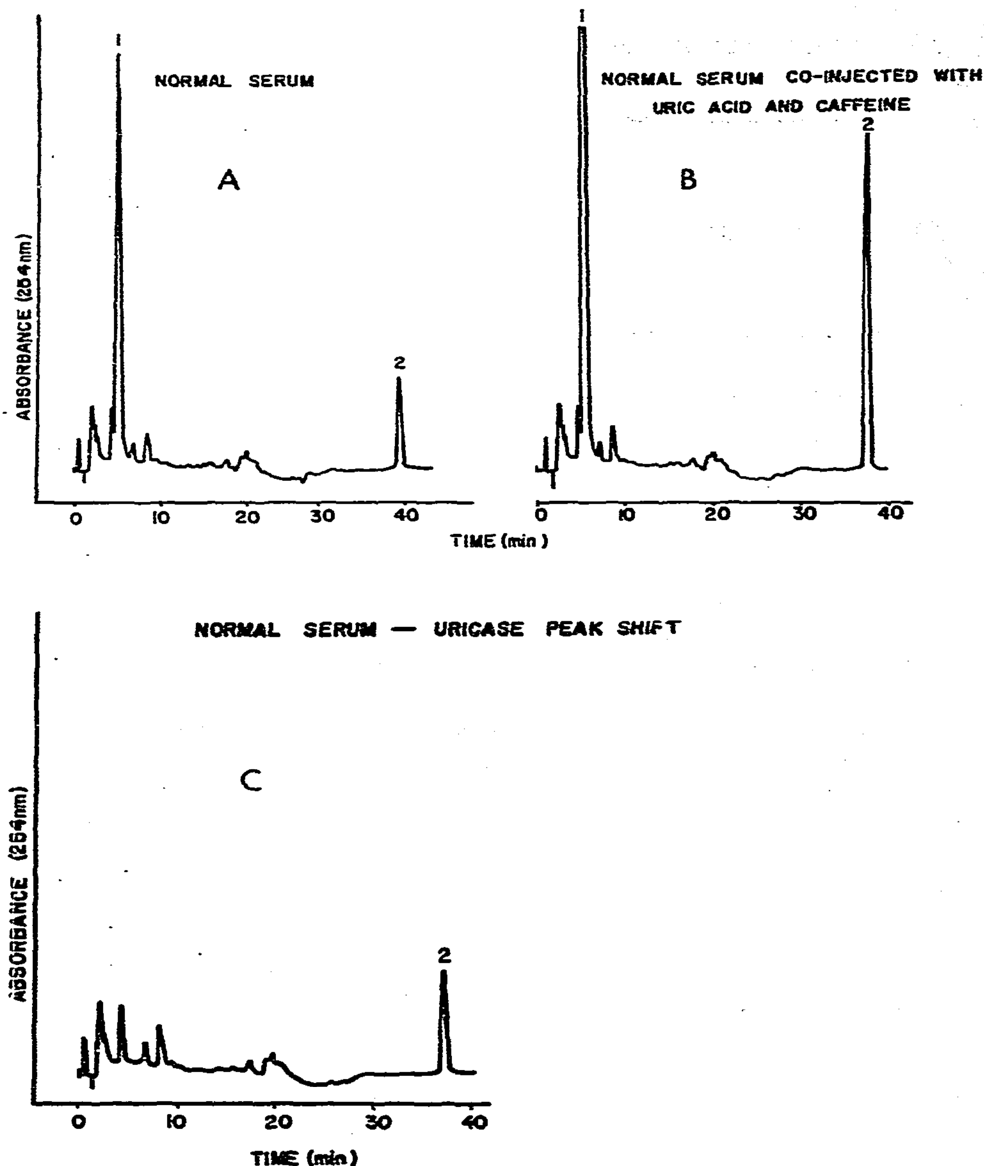


Fig. 6. (A) Chromatogram of a serum sample from a normal subject. (B) Chromatogram of a normal serum sample co-injected with uric acid. Chromatographic conditions as in Fig. 1. (C) Uricase peak shift carried out on a serum sample from a normal subject. Chromatographic conditions for all three chromatograms as in Fig. 1.

absorbance ratios (280/254 nm) that were obtained showed very close agreement, the average values of triplicate runs for peak 2 in normal serum and the caffeine standard being 1.51 $\pm$ 0.01 and 1.49 $\pm$ 0.01, respectively. Thus, it is concluded that the peak of interest is caffeine.

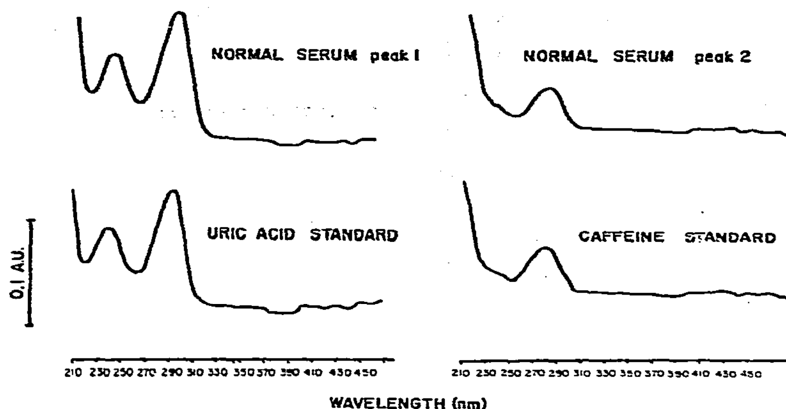
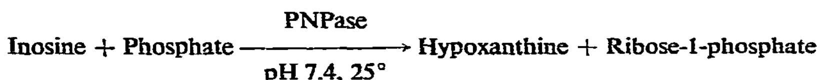


Fig. 7. UV spectra of peak 1 in normal serum and the uric acid standard, and of peak 2 in normal serum and the caffeine standard. Scanning conditions as in Fig. 2.

Identification of inosine is illustrated with a serum sample from a patient suffering from severe depression. The prominent peak in the middle of the chromatogram had the retention time of inosine (Fig. 8A). Therefore, the sample was co-chromatographed with the inosine standard and a net increase in the area of the peak under study indicated that the peak in serum was inosine (Fig. 8B). The full UV spectrum of the peak of interest was the same as that of inosine (Fig. 9). The absorbance ratios (280/254 nm) (average values of triplicate runs) for peak 1 in serum from the depressed patient and the inosine standard were 0.12 ± 0.01 and 0.11 ± 0.01, respectively. The sample was also incubated with a non-specific enzyme, purine nucleoside phosphorylase (PNPase), under the following conditions:



$$\text{Inosine} + \text{Phosphate} \xrightarrow[\text{pH 7.4, 25°}]{\text{PNPase}} \text{Hypoxanthine} + \text{Ribose-1-phosphate}$$

PNPase catalyzes the conversion of all nucleosides into their corresponding bases and provides evidence that the peak of interest was a purine nucleoside. The chromatogram of the incubated mixture (Fig. 8C) shows the conversion of the peak with the retention time of inosine into a peak with the retention time of hypoxanthine. Thus, from the accumulated evidence, it is concluded that the peak of interest is inosine.

Tryptophan, which is an essential amino acid, is very important because it has been implicated in breast cancer[17], bladder cancer[18,19] and Parkinson's disease[20,21]. With the ultrafiltration method of sample preparation, only the free tryptophan in serum is analyzed by HPLC. For the determination of the total tryptophan content of serum, TCA is used to deproteinize the serum in the sample preparation step. The chromatogram of the TCA extract of serum from a normal subject is shown in Fig. 10A and that of the extract co-injected with standard tryptophan in Fig. 10B. The UV spectra of the peak in the TCA extract and the tryptophan standard are shown in Fig. 11. The absorbance ratio of the peak of interest is in close agreement

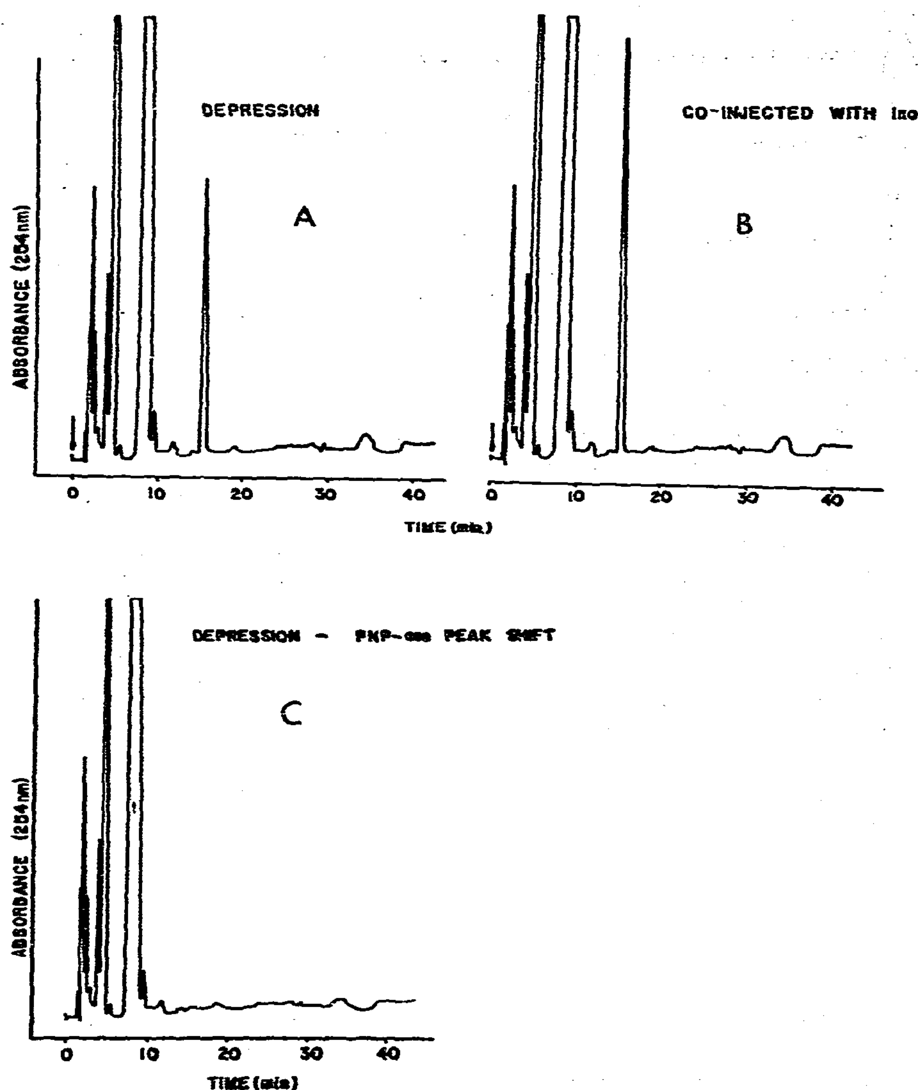


Fig. 8. (A) Chromatogram of a serum sample from a patient suffering from severe depression. (B) Chromatogram of a serum sample from a patient suffering from severe depression, co-injected with inosine. (C) Purine nucleoside phosphorylase peak shift carried out on a serum sample from a patient with severe depression. Chromatographic conditions for all three chromatograms as in Fig. 1.

with that of the standard. Thus, the UV spectrum gives substantial support in identifying the peak of interest as tryptophan.

In conclusion, the use of UV scanning techniques shows potential for characterizing components of complex biological mixtures, particularly as very accurate corrected spectra can be obtained from very small peaks. Used alone or in conjunction with absorbance ratios and/or enzymatic peak-shift techniques, UV scanning provides a simple, reliable, on-line method for the identification of peaks in HPLC

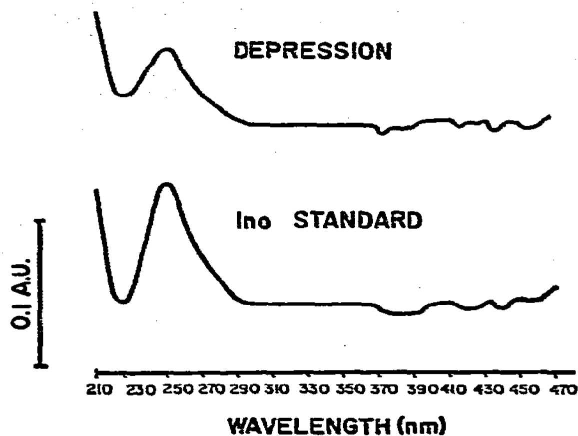


Fig. 9. UV spectra of peak 1 in the serum sample from a patient with severe depression and the nosine standard. Scanning conditions as in Fig. 2.

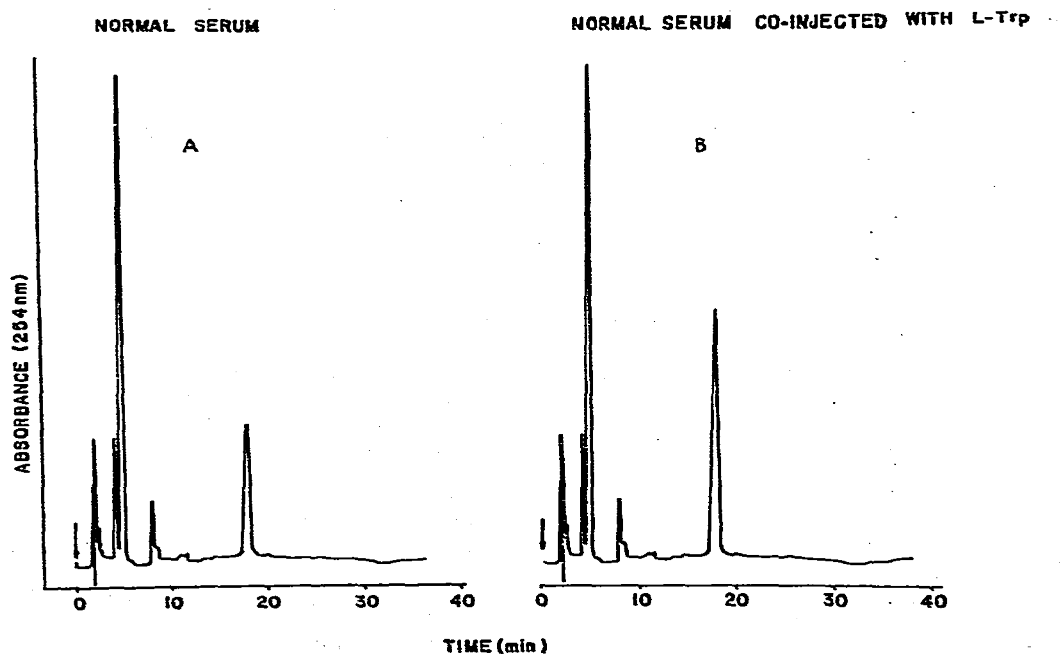


Fig. 10. (A) Chromatogram of the TCA extract of a serum sample from a normal subject. (B) Chromatogram of the TCA extract of normal serum sample co-injected with the tryptophan standard. Chromatographic conditions for both chromatograms as in Fig. 1.

eluates. Although its use has been illustrated only with serum constituents, the method is applicable to constituents in any biochemical samples provided that the compounds of interest absorb within the operating range of the detection system used.

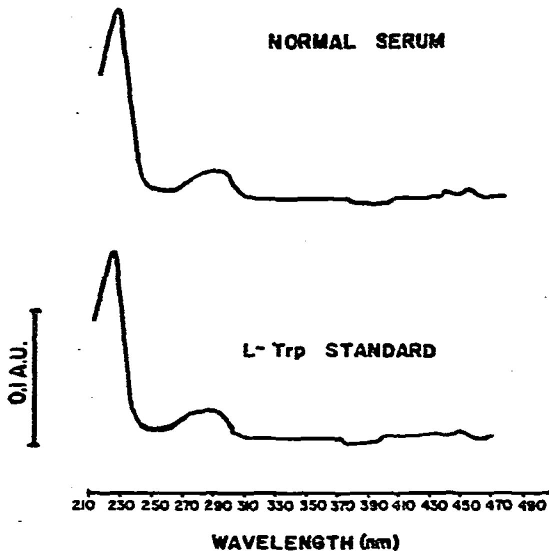


Fig. 11. UV spectra of peak 1 in the TCA extract of normal serum and the tryptophan standard.

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