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Note

Evaluation of combined electrochemical and ultraviolet detection in the high-performance liquid chromatographic analysis of plasma for low-molecular-weight constituents

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The development of high-performance liquid chromatography (HPLC) has made possible accurate and rapid high-resolution methods for the separation of the low-molecular-weight constituents in physiological samples, such as tissue, blood and urine [1—12]. However, with the high resolution supplied by HPLC, peak identification is difficult: thus different methods are needed to identify peaks unambiguously.

Electrochemical detection (ED) in liquid chromatography (LC) has become popular as a selective method of detection in trace organic analyses, especially for biologically active materials in complex matrices [13]. Among the advantages of LC—ED are excellent selectivity, high sensitivity, and flexibility due to the variety of available electrode materials. The detector is also compatible with post-column derivatization [14]. With the amperometric detector [15] the potential can be selected; thus sensitivity and selectivity can be maximized and interferences minimized or eliminated. Routinely picogram quantities of an analyte can be measured with femtogram detection limits.

In this investigation we studied the LC—ED behavior of the purine bases uric acid, xanthine, and hypoxanthine, the nucleoside guanosine, and the nucleotides xanthosine 5′-phosphate and guanosine 5′-phosphate as well as two important aromatic amino acids, tyrosine and L-tryptophan. These particular compounds were chosen because they represent the various types of compounds that are present in plasma. Both hypoxanthine and xanthine were selected because they are not sufficiently resolved in the HPLC profiles of blood fluids under the conditions routinely used in our laboratory. In addition, compounds such as the nucleotides, uric acid and tyrosine elute near the void volume and are difficult to resolve and identify. Although the electrochemical

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behavior of some of these molecules has been examined [16—19], no studies have been made of the HPLC separation, detection, and identification of all these compounds present in plasma which are important in biomedical studies.

The LC—ED analysis of these compounds was enhanced by optimizing chromatographic separations and several instrumental considerations. A dual-wavelength detector was used to ascertain when a compound was being eluted to the electrochemical detector.

EXPERIMENTAL

Instrumentation

The chromatographic instrumentation consisted of a Perkin-Elmer Series 10 liquid chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a Rheodyne 7125 injection port and a 20-μl injection loop. A Waters Model 440 dual-wavelength detector (280 and 254 nm) (Waters Assoc., Milford, MA, U.S.A.) and a Perkin-Elmer LC-4B electronic controller and LC-17 oxidative flow cell were utilized throughout the study. The working electrode was glassy carbon. The oxidation potential was maintained versus a silver/silver chloride reference electrode.

Peak heights at two wavelengths were recorded by means of a Fisher Series 5000 dual-pen strip chart recorder (Houston Instruments, Houston, TX, U.S.A.). Integration and retention times of electroactive compounds were obtained using a Hewlett-Packard Model 3390A electronic integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

All separations were performed on a prepacked 25 cm × 4 mm I.D. C_{18} column, Whatman Partisil 10 ODS-3. A 5-cm guard column packed with pellicular C_{18} material, particle size 30—37 μm (Whatman, Clifton, NJ, U.S.A.), was installed before the analytical column.

A schematic diagram of the analytical system utilized in this study is shown in Fig. 1. Note that the UV detector is placed prior to and in series with the electrochemical detector.

Reagents

Chromatographic standards of the purine derivatives and aromatic amino acids were obtained from Sigma (St. Louis, MO, U.S.A.) and were of the highest grade available. Distilled, deionized water, used for preparation of

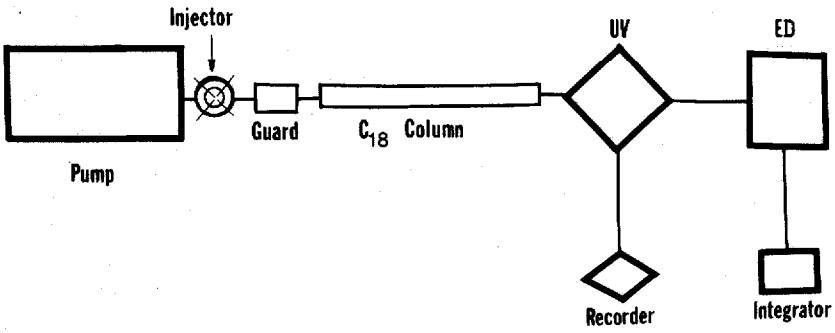

Fig. 1. Schematic diagram of the chromatographic system.

eluents and standard solutions, was filtered through membrane filters, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.). Distilled-in-glass methanol was obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Potassium dihydrogen phosphate was purchased from Fisher Scientific (Mallinckrodt, St. Louis, MO, U.S.A.).

Selection of chromatographic conditions

When selecting a mobile phase for LC—ED analyses, three important requirements must be met. First, the mobile phase must contain an electrolyte. This electrolyte must be sufficiently concentrated to minimize solution resistance. Secondly, if an organic modifier is to be added to the mobile phase, it must have a high enough dielectric constant to permit ionization of the electolyte. Finally, the composition of the mobile phase (electrolyte and organic modifier) must be electrochemically inert at the electrode surface.

The selection of mobile phase composition for this study was based on these three requirements. Upon experimentally varying the concentration of potassium dihydrogen phosphate from 0.01 to 0.10 *M*, a solution that was 0.02 *M* was found to give sufficient charging current. The methanol concentration was varied from 0 to 5% (v/v) in the 0.02 *M* potassium dihydrogen phosphate. It was found that addition of 3% methanol afforded adequate separation and reduced retention times so that relatively rapid analyses could be obtained.

Silica-based columns are stable in the pH range of 2—7.5. It has been found that in reversed-phase LC, the retention volume of a solute changes with pH or ionic strength only when the solute becomes ionized [6, 20]. At a pH of 5.7, good resolution was achieved for the majority of the compounds of interest.

The selection of a flow-rate of 1 ml/min was based on electrochemical detector response and maximum allowable resolution.

All separations were performed isocratically at ambient temperature. The eluent was 0.02 *M* potassium dihydrogen phosphate—3% methanol (v/v), adjusted to pH 5.7 using dilute potassium hydroxide solution. The flow-rate was 1.0 ml/min. Chromatographic standard solutions were prepared in 0.02 *M* potassium dihydrogen phosphate. All solutions were kept frozen when not in use. The minimum oxidation potential was determined under the chromatographic conditions used for each compound of interest to utilize the maximum selectivity in peak identification.

TABLE I

COMPONENTS OF STANDARD MIXTURE

Compound	Peak Number	Concentration (*M*)
Guanosine 5'-phosphate	1	$1.974 \cdot 10^{-6}$
Xanthosine 5'-phosphate	2	$2.648 \cdot 10^{-6}$
Uric acid	3	$2.037 \cdot 10^{-5}$
Tyrosine	4	$1.098 \cdot 10^{-7}$
Hypoxanthine	5	$4.949 \cdot 10^{-7}$
Xanthine	6	$9.218 \cdot 10^{-5}$
Guanosine	7	$1.030 \cdot 10^{-6}$
L-Tryptophan	8	$2.196 \cdot 10^{-7}$

RESULTS AND DISCUSSION

Chromatographic separations of a standard mixture of the selected nucleic acid constituents and the aromatic amino acids tyrosine and L-tryptophan were obtained using the isocratic mode of HPLC. The concentration of each component in a 20-μl aliquot of the mixture is shown in Table I. Using different applied potentials, chromatograms of the standard mixture were obtained. Examples of these chromatograms are shown in Fig. 2.

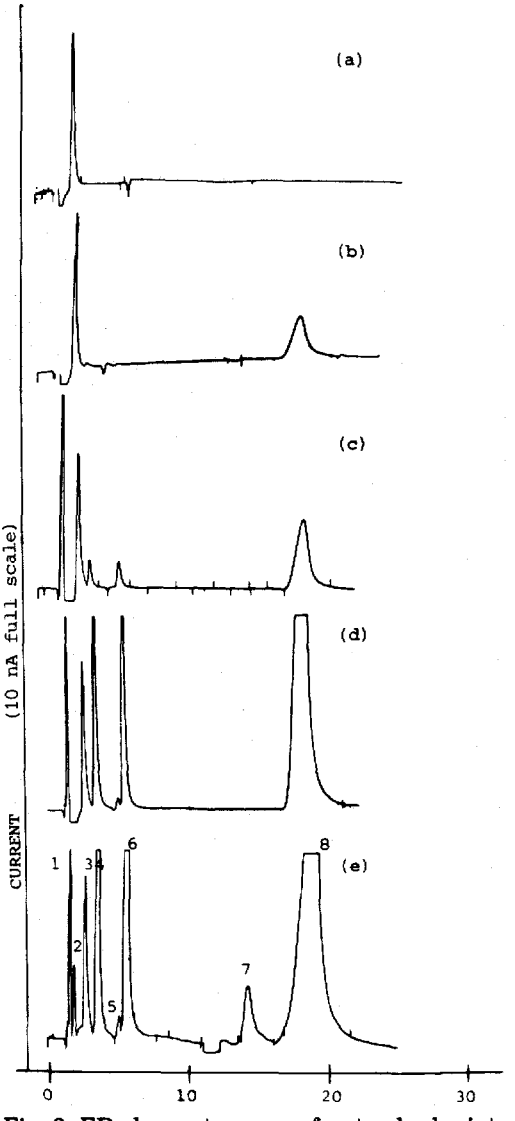

Fig. 2. ED chromatograms of a standard mixture at (a) +0.50 V, (b) +0.60 V, (c) +0.70 V, (d) +0.80 V, and (e) +0.90 V. Chromatographic conditions: flow-rate 1.0 ml/min; eluent 0.02 *M* potassium dihydrogen phosphate — 3% methanol (v/v), pH 5.7; 20-μl injections. Peaks: 1 = guanosine 5'-phosphate; 2 = xanthosine 5'-phosphate; 3 = uric acid; 4 = tyrosine; 5 = hypoxanthine; 6 = xanthine; 7 = guanosine; 8 = L-tryptophan.

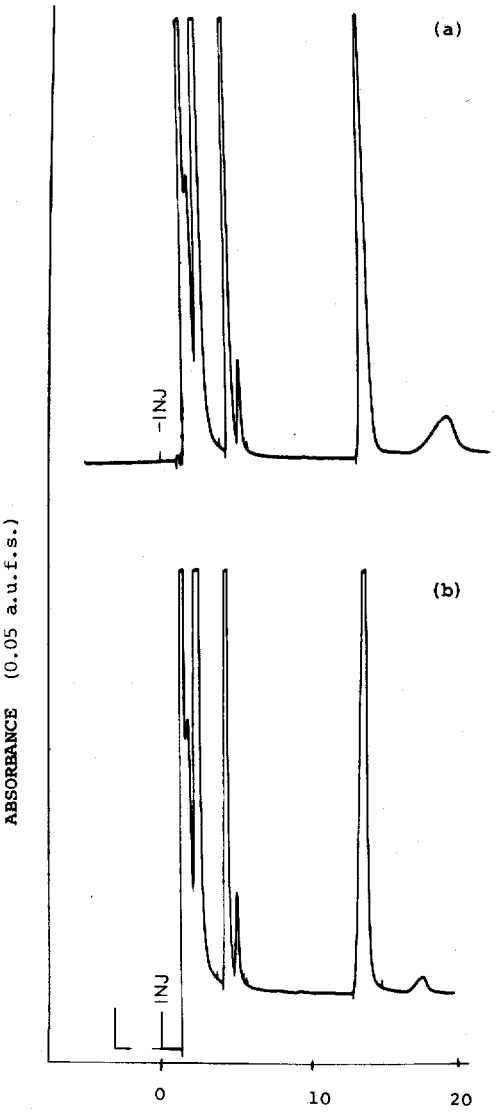

Fig. 3. UV chromatograms of a standard mixture monitored at (a) 254 nm and (b) 280 nm. Chromatographic conditions as in Fig. 2.

TABLE II

APPLIED POTENTIALS AND DETECTION LIMITS OF STANDARDS

Compound	Potential (V)	Detection limit (pg/ml)
Uric acid	+0.50	16
Tyrosine	+0.75	22
Guanosine	+0.95	250
Xanthine	+0.90	275
L-Tryptophan	+0.80	24
Hypoxanthine	+0.95	334
Guanosine 5′-phosphate	+0.75	42
Xanthosine 5′-phosphate	+0.95	374

As can be seen from these chromatograms, judicious selection of oxidation potential in the separation of these compounds is crucial. For example, at an applied potential of +0.50 V, the only component of the mixture detected under these conditions is uric acid. As the potential is raised, chromatographic peaks for the other compounds appear. By choosing the appropriate applied potential, interferences can be eliminated; thus the presence of one or several particular compounds can be selectively determined. When the potential is raised beyond +0.90 V, the electrochemical and chromatographic selectivity become inadequate to detect selectively the mixture components.

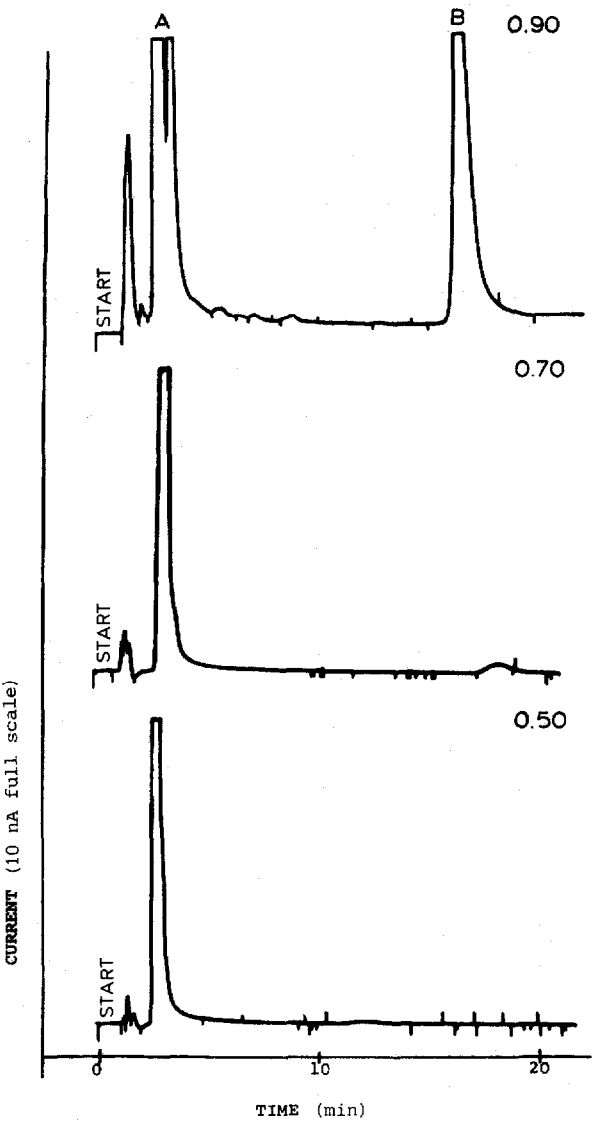

Fig. 4. ED chromatogram of human plasma at +0.90, +0.70 and +0.50 V. Chromatographic conditions as in Fig. 2. Peaks: A = uric acid; B = tryptophan.

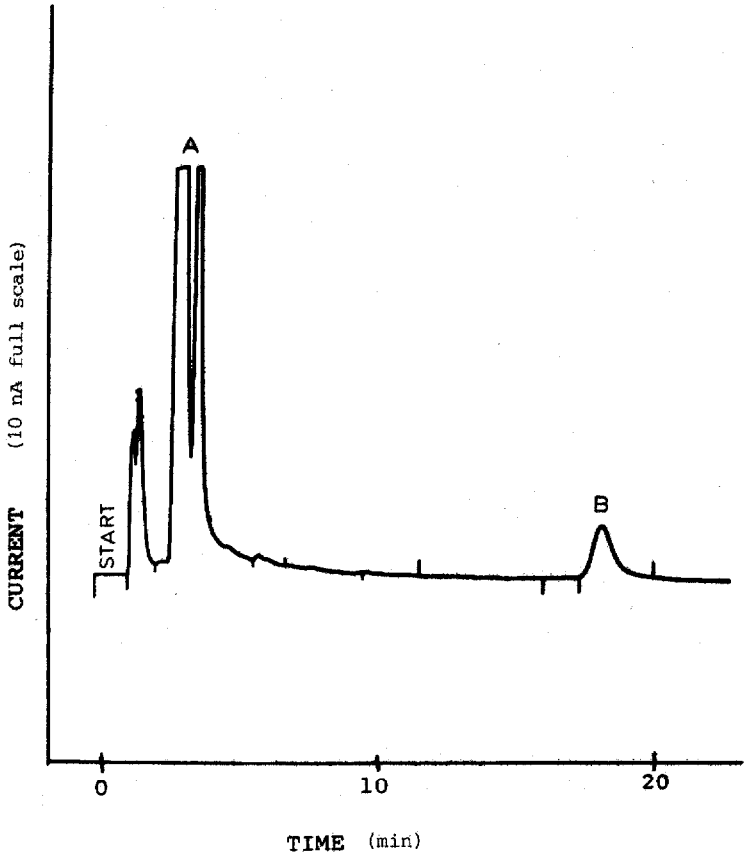

Fig. 5. ED chromatogram of human plasma ultrafiltrate at +0.90 V. Chromatographic conditions as in Fig. 2. Peaks: A = uric acid; B = tryptophan.

The UV chromatograms of the same mixture, using a dual-wavelength detector with the wavelengths set at 254 and 280 nm, are shown in Fig. 3. As can be seen from these two chromatograms, the components of the mixture absorbed in the UV region but they are not distinct peaks under these conditions. The resolution of peaks in these chromatograms is not as good as that shown in previous work [8—11] because isocratic elution was used with the electrochemical detector.

Detector response curves were generated for the electroactive compounds at the selected applied potentials. Linearity (typical correlation coefficient of 0.998) over the concentration range investigated (10^{-4} to 10^{-8} *M*) was observed. The lower limits of detection for this chromatographic system as well as the selected potentials for each of the compounds are listed in Table II.

Identification of the components of the standard mixture was made on the basis of retention times, enzymatic peak shifts, co-chromatography with standards, 280/254 UV ratios and on the basis of applied potentials.

Identification of peaks in complex matrices such as serum, plasma or urine is more difficult than identifying peaks in standard solutions because of the possibility of the presence of endogenous interferences or exogenous compounds.

However, ED can aid appreciably in the identification of peaks because both electrode and potential can be varied to minimize interferences and selectively detect certain constituents. For example, ED chromatograms of human plasma at +0.90, +0.70 and +0.50 V are shown in Fig. 4. At 0.50 V the presence of uric acid can be unequivocally identified. At 0.70 and 0.90 V, three other peaks can be observed. Positive identification of these peaks has not yet been made. Furthermore, the effect of ultrafiltration prior to chromatography is shown in Fig. 5 which is an ED chromatogram of human plasma ultrafiltrate at 0.90 V. The chromatograms are very similar but differences were noted in the first peak which is split in the ultrafiltrate but not in the plasma and in the size of the last peak. On the basis of retention times, electroactivity, absorbance ratios and the enzyme peak shift technique, the large peak (A) was tentatively identified as uric acid and the last peak (B) as tryptophan. The tryptophan peak in the plasma sample is much larger than that of the ultrafiltrate of the plasma because total tryptophan (free and bound) is measured in the plasma but only the amount of free tryptophan is determined in the ultrafiltrate. It should be noted that catecholamines may be present in plasma and these readily oxidize at potentials above 0.65 V.

CONCLUSIONS

The use of an amperometric detector in combination with a UV dual-wavelength detector shows promise for the routine analysis of selected low-molecular-weight constituents in physiological fluids by HPLC. This technique has the advantages of high sensitivity and selectivity. An electrochemical detector used on-line with an UV detector can be valuable in characterizing selected peaks of physiological fluids such as plasma and serum. Although the electrochemical detector can be useful because the applied potential can be selected to optimize detectability of the peak of interest and to minimize or eliminate interferences, a combination of several methods should be used for unambiguous identification of all the peaks in a chromatogram of a complex biological matrix.

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REFERENCES

1 P.R. Brown, J. Chromatogr., 52 (1970) 257.
2 R.A. Hartwick, S.P. Assenza and P.R. Brown, J. Chromatogr., 186 (1979) 645.
3 R.A. Hartwick, A.M. Krstulovic and P.R. Brown, J. Chromatogr., 186 (1979) 659.
4 J.H. Khym, Clin. Chem., 21 (1975) 1245.
5 G.E. Davis, R.D. Suits, K.C. Kuo, C.W. Gehrke, J.P. Waalkes and E. Borek, Clin. Chem., 23 (1977) 1427.
6 C.W. Gehrke, K.C. Kuo, G.E. Davis, R.D. Suits, J.P. Waalkes and E. Borek, J. Chromatogr., 150 (1978) 455.

7 A.C. Burtis, M.N. Munk and F.R. MacDonald, Clin. Chem., 16 (1970) 667.
8 A.M. Krstulovic, P.R. Brown and D.M. Rosie, Anal. Chem., 49 (1977) 2237.
9 A.M. Krstulovic, R.A. Hartwick, P.R. Brown and K. Lohse, J. Chromatogr., 158 (1978) 365.
10 P.R. Brown, R.A. Hartwick and A.M. Krstulovic, Adv. Chromatogr. (N.Y.), 18 (1980) 101–138.
11 R.A. Hartwick and P.R. Brown, CRC Crit. Rev., 10 (1981) 279.
12 M. Uziel, C.K. Koh and W.E. Cohn, Anal. Biochem., 25 (1968) 77.
13 R.E. Shoup (Editor), Recent Reports on Liquid Chromatography/Electrochemistry, BAS Press, West Lafayette, IN, 1982.
14 K. Shimada, M. Tanaka and T. Nambara, Chem. Pharm. Bull., 27 (1979) 2259.
15 P.T. Kissinger, C.J. Refshauge, R. Dreiling and R.N. Adams, Anal. Lett., 6 (1973) 465.
16 W.F. Smyth, (Editor), Polarography of Molecules of Biological Significance, Academic Press, New York, 1979.
17 G. Dryhurst and P.J. Elving, Talanta, 16 (1969) 855.
18 G. Dryhurst and P.K. De, Anal. Chim. Acta, 58 (1972) 183.
19 G. Dryhurst and B.H. Hansen, J. Electroanal. Chem. Interfacial Electrochem., 30 (1971) 417.
20 R.A. Hartwick and P.R. Brown, J. Chromatogr., 126 (1976) 679.