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Note

Rapid separation of tryptophan, kynurenines, and indoles using reversed-phase high-performance liquid chromatography

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In the course of our work involving the characterization of tryptophan degradation products which are formed when tryptophan is exposed to peroxidizing lipids or ionizing radiations in model systems, we have developed an efficient reversed-phase high-performance liquid chromatography (HPLC) method which quantitatively separates 3-hydroxykynurenine, kynurenine, N-formylkynurenine, tryptophan, 3-indoleacetic acid, 3-indolelactic acid, and tryptamine in approximately 24 min.

Tryptophan, an essential amino acid, is one of the most labile amino acid residues in proteins which have been exposed to peroxidizing lipids, ionizing radiations, or ultraviolet light[1]. The decomposition of tryptophan is associated with the yellowing of wool[2], with the development of yellow and brown cataracts in the lens of the human eye[3], and with off-flavors in irradiated foods[4]. N-Formylkynurenine, a tryptophan decomposition product, is a powerful photodynamic sensitizer for a great variety of biological compounds[5]. Various metabolites of tryptophan, such as serotonin, 3-indoleacetic acid, and tryptamine, exhibit strong biological activities in animals or plants[6]. Clinically, it has been observed that large amounts of 3-hydroxykynurenine and kynurenine are excreted in the urine of patients with various blood[7] and metabolic[8] disorders as well as cancer[9]. Thus, a method for rapid separation of tryptophan and its derivatives would provide an important and useful analytical tool for a wide spectrum of research areas.

EXPERIMENTAL

Reagent grade chemicals were used in this work without further purification. L-Tryptophan, tryptamine monohydrochloride, L-kynurenine, L-3-hydroxykynurenine, 3-indoleacetic acid, and N-formylkynurenine were purchased from Calbiochem (San Diego, Calif., U.S.A.). 3-Indolelactic acid was obtained from Sigma (St. Louis, Mo., U.S.A.) and potassium phosphate (monobasic) and phosphoric acid were purchased from J. T. Baker (Phillipsburg, N.J., U.S.A.).

Our reversed-phase HPLC system consisted of a Waters Assoc. (Milford,

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Mass., U.S.A.) 6000A pump, a Waters Model 440 UV absorbance detector, a Valco 7000 p.s.i.g.-injector equipped with a 25 μl loop (Valco instruments, Houston, Texas, U.S.A.), and an Omniscribe B-5000 strip chart recorder (Houston Instruments, Austin, Texas, U.S.A.). The column, μBondapak C_{18} 30 cm × 3.9 mm I.D.; Waters Assoc., was purchased prepacked; a Waters guard column (2 cm × 3.9 mm I.D.) containing Bondapak C_{18}–Corasil was attached between the injector and the analytical column to extend the life of the analytical column. The mobile phase consisted of different mixtures of methanol (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.) and distilled, buffered (0.001 *M* KH_2PO_4, adjusted to pH 4 with phosphoric acid) or unbuffered water. All aqueous methanol solutions were filtered through 0.6-μm Millipore (Bedford, Mass., U.S.A.) Polyvic filters and de-aerated by sonication prior to use. Typically, 25 μl of water–methanol solution (90:10) containing standard mixtures of tryptophan and its derivatives was injected into the column and the separated compounds were monitored at 254 nm with an a.u.f.s. setting of 0.1. Chromatography was performed at ambient temperature with a flow-rate of 1.8 ml/min.

RESULTS AND DISCUSSION

Of the solvent systems studied, we found that a stepwise elution technique, using phosphate buffer (0.001 *M*, pH 4)–methanol (90:10), unbuffered water–methanol (90:10), and phosphate buffer (0.001 *M*, pH 4)–methanol (75:25), yielded a good separation of all the seven compounds in the mixture in less than 25 min (Fig. 1). When unbuffered water–methanol (90:10) was used as the only mobile phase, 3-hydroxykynurenine, kynurenine, N-formylkynurenine, and tryptophan could be separated in approximately 8 min. This is a significant improvement over the method reported by Grushka *et al*[10]. According to their method, it took 12 min to separate these four compounds, and there was an overlapping of kynurenine and tryptophan peaks, so that the researchers had to monitor absorptions at 254 and 289 nm simultaneously in order to determine tryptophan concentration (because kynurenine does not absorb at 280 nm).

Unfortunately, our simple mobile phase (unbuffered water–methanol, 90:10) could not separate a more complex mixture of indoles and kynurenines. When 3-indoleacetic acid, 3-indolelactic acid and tryptamine were added to the mixture containing the previously mentioned four compounds, 3-indolelactic acid was not completely separated from 3-hydroxykynurenine, 3-indoleacetic acid overlapped with N-formylkynurenine and tryptamine could not be eluted from the column. Therefore, we decided to increase the retention times of 3-indoleacetic and 3-indolelactic acids by using a phosphate buffer (0.001 *M*, pH 4)–methanol solution (90:10) as the initial mobile phase. At pH 4, these acidic compounds become less ionic and are better retained by the non-polar stationary phase because the equilibrium prefers $-COOH$ over $-COO^-$. Separation of 3-hydroxykynurenine, kynurenine, N-formylkynurenine, and tryptophan was only slightly affected by the buffer at pH 4, probably due to the presence of both $-COO^-$ and $-NH_4^+$ groups in these compounds. The presence of buffer somewhat reduced tailing in N-formylkynurenine and tryptophan peaks. Tryptamine, along with 3-indoleacetic and 3-indolelactic acids, was retained in the column upon elution with the phosphate buffer–methanol solution

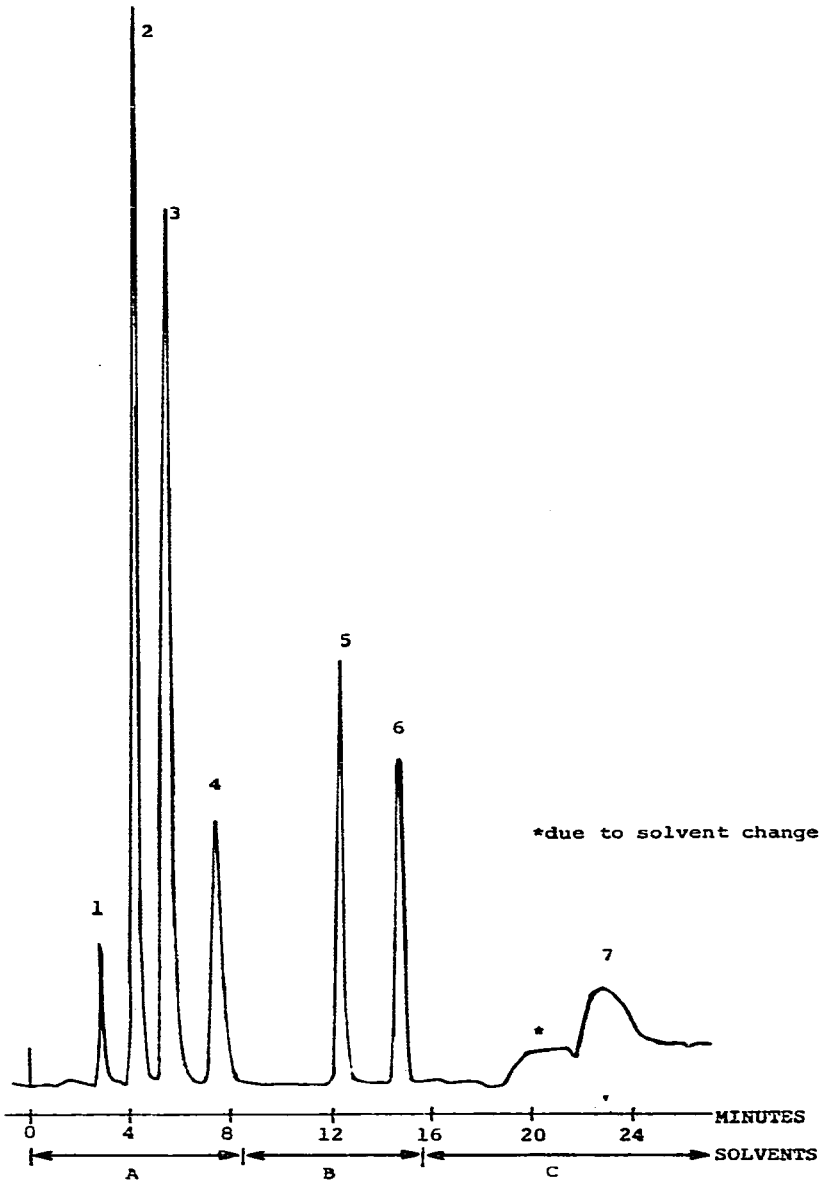

Fig. 1. Separation of tryptophan and its derivatives by reversed-phase HPLC. Column: μBondapak C_{18}, 30 cm × 3.9 mm I.D. Solvent: stepwise elution with (A) phosphate buffer (0.001 *M* KH_2PO_4, pH 4)–methanol (90:10), (B) unbuffered water–methanol (90:10), (C) phosphate buffer (0.001 *M* KH_2PO_4, pH 4)–methanol (75:25). Sample: 25 μl of water–methanol solution (90:10) containing approximately 1.4 μg each of (1) 3-hydroxykynurenine, (2) kynurenine, (3) N-formylkynurenine, (4) tryptophan, (5) 3-indolelactic acid, (6) 3-indoleacetic acid, and (7) tryptamine. Flow-rate: 1.8 ml/min. Detector: UV, 254 nm, 0.1 u.a.f.s.

(90:10). When the elution strength of the mobile phase was increased stepwise by using a phosphate buffer–methanol solution (80:20), all three compounds were eluted in poorly resolved peaks. 3-Indolelactic and 3-indoleacetic acids, which were retained in the column during elution with the first mobile phase, could be eluted as

separate peaks by using unbuffered water–methanol (90:10) as the second mobile phase. Tryptamine could not be eluted by simply increasing the concentration of methanol in unbuffered water–methanol mixtures, but it was successfully eluted by using phosphate buffer (0.001 *M*, pH 4)–methanol solution (75:25) as the third mobile phase.

Our research has demonstrated the usefulness of buffer–organic solvent mixtures in improving the resolution of a reversed-phase HPLC method. At the same time, we have shown that, in addition to simply increasing the elution power by adding more organic solvent to the mobile phase, chromatographic separations can sometimes be improved by using stepwise elution techniques which consist of buffer–organic, unbuffered water–organic, and buffer–organic mobile phases.

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REFERENCES

1 M. Karel and S. H. Yong, in *Proceedings, Second International Symposium on Properties of Water in Relation to Food Quality and Stability*, Academic Press, New York, in press.
2 F. G. Lennox and R. J. Rowlands, *Photochem. Photobiol.*, 9 (1969) 359.
3 A. Pirie, *Biochem. J.*, 125 (1971) 203.
4 M. Friedman and J. W. Finley, in M. Friedman (Editor), *Protein Nutritional Quality of Foods and Feeds*, Part 1, Marcel Dekker, New York, 1975, p. 423.
5 P. Walrant and R. Santus, *Photochem. Photobiol.*, 19 (1974) 411.
6 B. B. Stowe, *Progr. Chem. Org. Nat. Prod.*, 17 (1959) 249.
7 L. Musajo, C. A. Benassi and A. Parbajola, *Nature (London)*, 175 (1955) 855.
8 J. H. French, R. B. Grueter, R. Druckman and D. O'Brien, *Neurology*, 15 (1965) 101.
9 R. R. Brown, J. M. Price, E. J. Satter and J. B. Wear, *Acta Unio Int. Contra Cancrum*, 16 (1960) 299.
10 E. Grushka, E. J. Kikta, Jr. and E. W. Naylor, *J. Chromatogr.*, 143 (1977) 51.