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Note

Purity control of riboflavin-5′-phosphate (vitamin B_2 phosphate) by capillary zone electrophoresis

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The control of the specified purity of commercial chemicals is an important task in chemical analysis. Often high demands are made on the analytical method because in many instances chemically very similar constituents have to be determined. For riboflavin-5′-phosphate the purity of the products investigated is specified by the producers based on high-performance liquid chromatography (HPLC) or spectrophotometry. Because the most important impurities are riboflavin and the different riboflavin phosphates, the latter method has the disadvantage that the particular riboflavin derivatives (showing similar optical properties) cannot be determined individually, and their total is measured. Therefore, this is not a very suitable technique for the determination of such impurities. Apart from the note on the product label refering to the analytical method applied, no detailed procedures concerning the purity control of riboflavin-5′-phosphate were found in the literature. Only its determination in different matrices, *e.g.*, in pharmaceutical preparations, drinks, foodstuffs, plasma, urine, animal tissues etc., has been described[1–12]. Capillary zone electrophoresis, which has a high separation efficiency, seems to be a very suitable method for the determination of impurities in riboflavin-5′-phosphate. Using conditions under which electroosmotic flow occurs, it is possible to control the purity of riboflavin-5′-phosphate in the presence not only of other ionic but also of neutral sample components such as riboflavin.

EXPERIMENTAL

Chemicals

All chemicals used for the preparation of the buffers were of analytical-reagent grade (E. Merck, Darmstadt, F.R.G.) and were used without further purification. Water was distilled twice from a quartz apparatus.

Riboflavin-5′-phosphate and riboflavin were purchased from different sources (Merck; Serva, Heidelberg, F.R.G.; Fluka, Buchs, Switzerland). The purity of the

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TABLE I

BUFFERING ELECTROLYTES WITH DIFFERENT pH VALUES USED FOR THE ZONE ELECTROPHORETIC SEPARATIONS

pH	*Composition*	*Concentration (mol/l)*
6.0	Morpholinoethanesulphonic acid–histidine	0.01
7.0	Sodium phosphate	0.01
8.2	Sodium phosphate	0.01
9.1	Sodium borate–boric acid	0.01

riboflavin-5′-phosphates was specified as follows: "for biochemistry", 99–101% (Merck); "pure" (Serva); and >89% (Fluka).

The samples were dissolved in the buffer electrolytes at a concentration of about 0.5 mg/ml. The compositions of the buffering electrolytes are given in Table I.

Apparatus

Zone electrophoresis was carried out on a laboratory-made instrument, consisting of a fused-silica separation capillary (100 cm × 100 μm I.D.) (Scientific Glass Engineering, Ringwood, Victoria, Australia), which was mounted in an HPLC fluorescence detector (Model F-1050, Merck-Hitachi). The high-voltage power supply unit (Tachophor 2020; LKB, Bromma, Sweden) was operated in the constant-voltage mode at 17.5 kV. Injection was carried out by electromigration with 10 kV for 5 s. The detector signal was recorded by an integrator (Model D-2500; Merck).

RESULTS AND DISCUSSION

Variation of the pH of the buffering electrolyte

Two parameters, which are decisive for the electromigration of the sample components, are affected by varying the pH of the buffer electrolyte: the electroosmotic flow of the bulk liquid, which is directed towards the cathode under the given conditions, and contributes to the migration of all components to the same extent, independent of the sign and amount of their charge; and the electrophoretic migration of the substances, which is dependent on the pH of the buffer electrolyte via the degree of dissociation.

The different electrophoretic patterns of a riboflavin-5′-phosphate sample, obtained at different pH values of the buffer, are shown in Fig. 1. There is an increase in the migration time of the analytes with decrease in pH. The longer migration time of the first-eluting substance (neutral riboflavin, detected, *e.g.*, at 5.02 min at pH 9.1 or at 7.18 min at pH 6), but also of the other analytes, is caused by the reduction in the electroosmotic flow: lowering the pH leads to a decrease in the number of negative charges on the silica surface (which originate from the dissociation of silanol groups) and therefore reduces the zeta potential. It follows that a high pH is of advantage when the time of analysis is of interest.

On the other hand it can be seen from the electropherograms in Fig. 1 that the separation of the analytes is enhanced on lowering the pH of the buffer. At pH 8.2 one

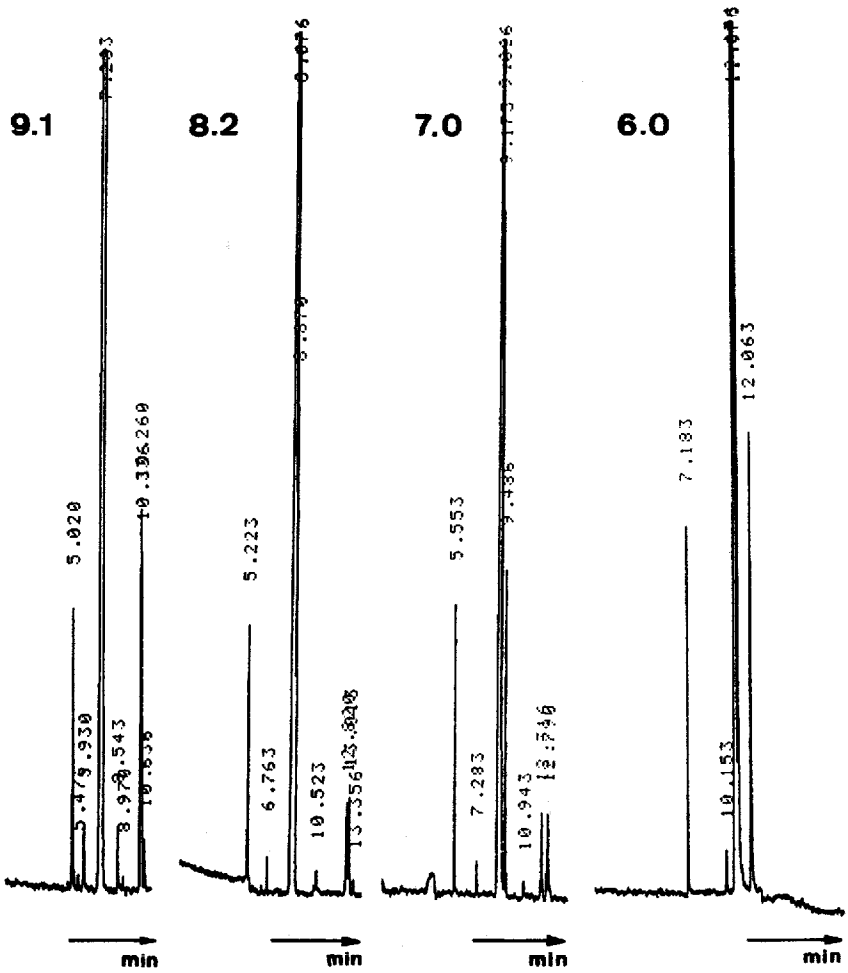

Fig. 1. Electropherograms of a sample of riboflavin-5′-phosphate at different pH values of the buffering electrolyte. Capillary: fused silica, 100 cm × 100 μm I.D., 55 cm distance to detector. Detection: fluorescence (350/520 nm). pH values are indicated by the numbers at the top of the electropherograms. The compositions of the buffers are given in Table I. The main peak is riboflavin-5′-phosphate; the first-eluting compound (*e.g.*, with a migration time of 5.02 min at pH 9.1) is riboflavin. Components with migration times longer than that of riboflavin-5′-phosphate are other riboflavin mono- and diphosphates.

additional peak (at 8.87 min) and a pH 7.0 two additional peaks (at 9.17 and 9.48 min, respectively) are resolved from the largest peak (riboflavin-5′-phosphate). The improved resolution is caused by the increase in selectivity due to changes in the effective mobilities of the analytes, caused by the different extents of dissociation.

Under the given conditions these components migrate as anions like the main component, riboflavine-5′-phosphate, showing similar fluorescence characteristics. Because of the nearly identical mobilities of the fully dissociated species, which are observed at high pH (9.1), it can be assumed that all these analytes are riboflavin

monophosphates, being isomers with respect to the position of the phosphate group on the saccharide.

A further decrease in the pH to 6.0 results in a better separation of the monophosphates, but the two anionic components with migration times of about 12 min at pH 7.0 (according to their electrophoretic behaviour they are probably riboflavin diphosphates) cannot be determined within a reasonable analysis time, because at pH 6 their electrophoretic velocities are too high compared with the electroosmotic velocity of the bulk liquid. It can therefore be concluded that the buffer of pH 7 will give the best results for quantitative analysis.

Precision of the migration time

The reproducibility of the migration times in capillary electrophoresis often depends on the nature of the sample. Especially with proteins, poor reproducibility is observed for subsequent injections, caused by changes in the electroosmotic flow due to interactions of sample components with the silica surface of the capillary, leading to variations of the zeta potential. In these instances, the reproducibility is enhanced by regeneration of the surface on rinsing the capillary with sodium hydroxide solution.

However, for the riboflavin-5′-phosphate samples no such interactions were observed. This is reflected by the excellent precision of the migration times obtained even without rinsing the capillary after the analytical run; a value of 0.6% was found, *e.g.*, for the relative confidence interval of the migration time of riboflavin-5′-phosphate at pH 9.1 (relative standard deviation 1.2%, 16 measurements). This is a typical value found with short term usage.

Quantification

Normalization of the analyte concentrations to 100% is usually applied for the specification of the purity of chemicals or drugs. In general, this method of calibration requires the determination of the individual response factors of the particular analytes. However, for the special analytical problem considered it can be assumed that the different riboflavin derivatives are all detected with equal response. Therefore, no individual calibration functions need to be determined.

When injection is carried out using electromigration, the components are not transferred into the separation capillary with the same mass ratios as in the original sample. Discrimination occurs, whereby the injected amounts are proportional to the sum of the velocity vectors of the electroosmotic and the electrophoretic migration. As electroosmosis causes a flow that is directed towards the cathode under given conditions, the resulting velocity for anions is given by the differences in the scalar velocities of electrophoresis and electroosmosis. The relative amounts of anionic analytes in the separation capillary are therefore always lower than those in the original sample. However, this discrimination by injection is counterbalanced, at least when the concentration of the sample components is significantly lower than that of the buffer in which they are dissolved, by the different migration velocities through the detector cell, which are also proportional to the sum of the vectors mentioned. Given equal fluorescence properties of the analytes, the peak areas (which are based on time, but not on length) can therefore be used directly for quantification.

With this assumption, the content of riboflavin-5′-phosphate was found to be between 72 and 75% in all samples, with a typical value of 1.5% for the relative

confidence interval (five measurements, 95% confidence level). The difference from 100% is caused by the presence of riboflaving (about 7%), other riboflavin monophosphates (about 16%) and riboflavin diphosphates (about 4%).

The deviations of the riboflavin-5′-phosphate contents from those given by the producers can be attributed to the different methods of determination. With spectrophotometric measurements the sum of all riboflavin derivatives and not riboflavin-5′-phosphate specifically is determined. The differences in the quantitative results obtained by (RP-)HPLC and capillary zone electrophoresis probably occur because in HPLC, in contrast to electrophoresis, only a group separation is achieved. Riboflavin and the riboflavin mono- and diphosphates can easily be separated, but the particular monophosphates are probably not resolved. Therefore, their individual contents are not determined.

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