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Note

High-performance liquid chromatographic determination of sugar phosphates and sugar acids, applied to the oxidation of glucose 1-phosphate

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Oxidation of glucose 1-phosphate (α-D-glucopyranosyl phosphate, I), by means of oxygen and a supported platinum catalyst yields mainly glucuronic acid 1-phosphate (α-D-glucopyranuronic acid 1-phosphate, II). Hydrolysis results in the formation of inorganic phosphate (III), glucose (IV) and glucuronic acid (VI). The last two products are rapidly oxidised to gluconic acid (V) and glucaric acid (VII), respectively. In the course of our investigation of the oxidation of I, we required a simple method for the determination of I–III, V and VII.

Anion-exchange chromatography has been used for the separation of phosphate esters[1–4]. However, in most instances complex salt and pH gradients had to be applied, which necessitated post-column colorimetric detection. I and III can be separated in an isocratic system[5], but broad peaks are obtained, and application of this method to the analysis of the complex oxidation mixtures seemed not to be feasible. In our laboratory, ion-pair reversed-phase chromatography has been used for the determination of I and II in oxidation mixtures[6]. Although a baseline separation can be obtained, this method is less practical when large numbers of samples, or small sample volumes, are involved. Ion-moderated partitioning (IMP) chromatography has been used for the determination of I in the presence of fructose and sucrose[7] and for the analysis of sugar acids[8].

We have reinvestigated the application of IMP chromatography to the analysis of I–III, V and VII. A cation-exchange resin in the hydrogen form was used as the stationary phase. In order to determine the scope of this method, the investigation also included D-glucose 6-phosphate (VIII) and D-fructose 6-phosphate (IX).

To avoid corrosion problems[7], the aqueous mobile phase was acidified with trifluoroacetic acid (TFA) rather than sulphuric acid. TFA is a moderately strong acid; the literature provides pK_a values ranging from 0.5 up to 1.1[9,10]. As this range is wide, the dissociation of TFA in the mobile phases used in this work was calculated (more directly) from pH measurements.

EXPERIMENTAL

Chemicals

Glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate (dipotassium

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salts) and TFA were obtained from Janssen Chimica (Beerse, Belgium). Glucuronic acid was purchased from Fluka (Buchs, Switzerland). Glucuronic acid 1-phosphate (tripotassium salt, pentahydrate) and monopotassium glucarate were prepared in our laboratory. Other chemicals were obtained from Merck (Darmstadt, F.R.G.). The compounds were injected as salts; lactones were not present in the samples.

Aminex A-7 sulphonic acid resin (polystyrene–8% divinylbenzene, 9 ± 2 μm) was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Chromatography

The chromatographic system consisted of the following elements: a Waters Assoc. M45 pump, a Perkin-Elmer ISS-100 autosampler, a slurry-packed[11] Aminex A-7 column (200 × 9 mm I.D.) and a thermostated Waters Assoc. R401 refractive index detector. The flow-rate was 0.4 ml/min. A faster flow-rate resulted in a less efficient separation and a decrease in the flow-rate gave no improvement. The column temperature was kept constant at 35°C. Higher temperatures resulted in significant hydrolysis of I when 0.2 *M* TFA was used as the mobile phase.

Owing to difficulties in defining and reliably measuring the void volume of the ion-exchange resin column, the results are presented in terms of retention times instead of capacity factors.

System peaks and sample preparation

When a high TFA concentration in the mobile phase was used (*e.g.*, 0.2 *M*), direct injection of reaction mixtures (or water) resulted in a large negative system peak. This peak, caused by the absence of TFA from the samples, interfered with the peak of I. Therefore, the samples were made 0.2 *M* in trifluoroacetate by the addition (Finn pipette) of concentrated aqueous potassium trifluoroacetate. This correction eliminated the negative peak without affecting the neutral pH of the samples.

When the corrected system was used and the TFA concentration in the mobile phase exceeded 0.01 *M*, chromatograms of samples containing (moderately) strong acids (*e.g.*, sugar phosphates) exhibited a small positive TFA peak. We ascribe this effect to the expulsion of undissociated TFA from the mobile phase into the stationary phase.

Glycerol was added to the samples as an internal standard for quantitative analysis (retention time 20 min).

pH measurements and dissociation calculations

A Cole Parmer standard combined glass electrode was used for pH measurements in TFA solutions (mobile phase). The electrode was calibrated at pH 2.00 and checked with 0.1 *M* hydrochloric acid (observed pH 1.085; calculated[12] pH 1.08). The extended Debye–Hückel equation[12] was used to calculate the activity coefficients, f_{H^+}. The results are given in Table I, together with the calculated H^+ concentrations and the extent of dissociation of TFA.

RESULTS AND DISCUSSION

Fig. 1 shows the retention times of the compounds under investigation *versus* the TFA concentration in the mobile phase. In all instances an increase in retention

TABLE I

pH VALUES, H^+ ACTIVITY COEFFICIENTS AND DISSOCIATION OF AQUEOUS TRIFLUOROACETIC ACID

[TFA] (M)	*pH**	*f_{H^+}***	*$[H^+]$*** (M)*	*Dissociation (%)*
0.20	0.88	0.80	0.165	83
0.10	1.125	0.83	0.090	90
0.050	1.385	0.86	0.048	96
0.010	2.04	0.91	0.010	100

* Measured (20°C).
** Calculated[12].
*** Log $[H^+] = -pH - \log f_{H^+}$.

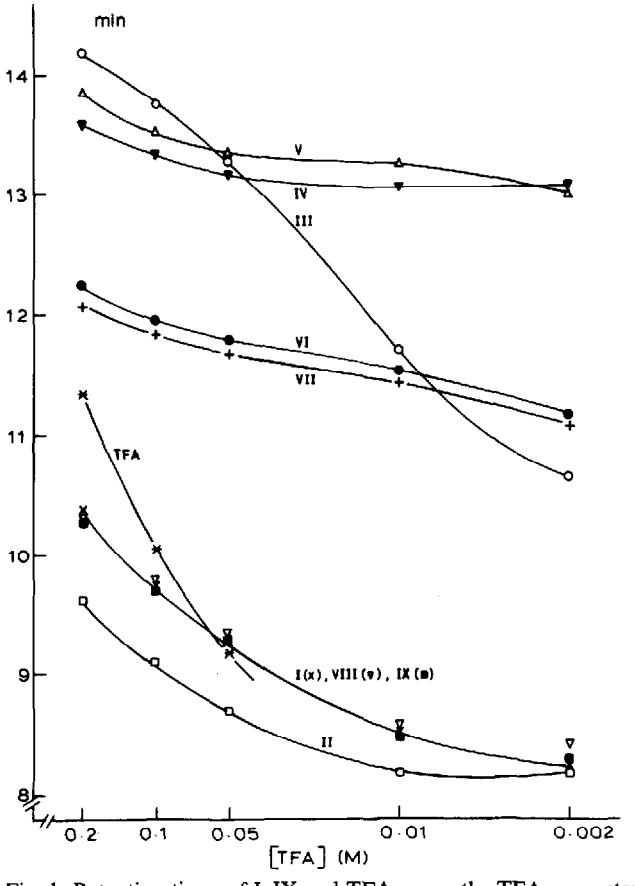

Fig. 1. Retention times of I–IX and TFA *versus* the TFA concentration in the mobile phase: I, glucose-1-phosphate; II, glucuronic acid 1-phosphate; III, inorganic phosphate; IV, glucose; V, gluconic acid; VI, glucuronic acid; VII, glucaric acid; VIII, glucose-6-phosphate; IX, fructose-6-phosphate; TFA (system peak).

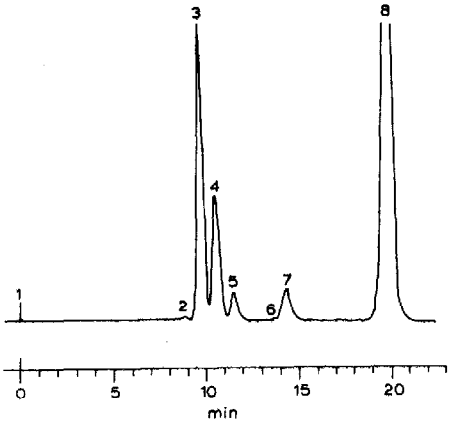

Fig. 2. Chromatographic analysis of oxidation mixtures: Peaks: 1 = injection; 2 = solvent front; 3 = glucuronic acid 1-phosphate (II); 4 = glucose-1-phosphate (I); 5 = system peak (TFA); 6 = gluconic acid (V); 7 = inorganic phosphate (III); 8 = glycerol (internal standard).

is observed at the highest TFA concentrations (0.05–0.2 *M*). This effect is probably connected with the dissociation of TFA, which is incomplete below pH 2 (Table I).

The internal H^+ concentration in the stationary phase (sulphonic acid resin) is approximately 1.7 *M*. In such media, the investigated compounds exist solely as undissociated acids. This implies that the retention of the acidic compounds increases when their dissociation in the mobile phase decreases. The sugar acids (gluconic, glucuronic and glucaric acid) have $pK_{a,1}$ values of about 3.6[9,10]. They are still partially dissociated in 0.002 *M* TFA (pH 2.7), but are virtually undissociated in the more concentrated mobile phases. The sugar phosphates are stronger acids ($pK_{a,1}$ = 1–1.5[9,10]) and are almost completely ionized in 0.002 *M* TFA. Retention of these compounds starts at pH 2, and increases rapidly in more acidic mobile phases. The TFA system peak, which appears only when the more concentrated mobile phases are used, shows the same behaviour (TFA, pK_a 0.5–1.1[9,10]; *cf.*, Table I). As expected, phosphoric acid ($pK_{a,1}$ = 2.1) shows an increased retention around 0.01 *M* TFA (pH 2).

As can be concluded from Fig. 1, samples taken during the oxidation of I can be analysed for I and II when 0.2 *M* TFA is used as the mobile phase. An example is given in Fig. 2. A near-baseline separation of I, II and the TFA system peak is obtained. Quantitative peak height analysis is possible (accuracy 3% absolute for 50 m*M* samples). III, V and VII can be analysed when 0.02 *M* TFA is used. I, VIII and IX cannot be separated from each other.

REFERENCES

1 M. H. Simatupang, *J. Chromatogr.*, 180 (1979) 177.
2 S. P. Bessman, P. J. Geiger, T.-C. Lu and E. R. B. McCabe, *Anal. Biochem.*, 50 (1974) 533.
3 H. J. Duncan, *J. Chromatogr.*, 62 (1971) 391.
4 P. J. Geiger, S. Alm and S. P. Bessman, *Methods Carbohydr. Chem.*, 8 (1980) 21.
5 K. Brunt and H. Hokse, *J. Chromatogr.*, 268 (1983) 131.
6 T. T. Tjioe, J. P. van der Wiel, R. M. Stikkelman, A. J. J. Straathof and F. van Rantwijk, *J. Chromatogr.*, 330 (1985) 412.

7 R. M. Stikkelman, T. T. Tjioe, J. P. van der Wiel and F. van Rantwijk, *J. Chromatogr.*, 322 (1985) 220.
8 K. B. Hicks, P. C. Lim and M. J. Haas, *J. Chromatogr.*, 319 (1985) 159.
9 G. Kortüm, W. Vogel and K. Andrusson, *Dissociationskonstanten Organischer Säuren in Wässeriger Lösung*, Butterworths, London, 1961.
10 E. P. Serjeant and B. Dempsey, *Ionisation Constants of Organic Acids in Aqueous Solution*, IUPAC Chemical Data Series, 23, Pergamon Press, Oxford, 1979.
11 L. A. Th. Verhaar and B. F. M. Kuster, *J. Chromatogr.*, 210 (1981) 279.
12 J. Kielland, *J. Am. Chem. Soc.*, 59 (1937) 1675.