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High-performance liquid chromatographic and mass spectrometric identification of phosphocitrate synthesized by human kidney homogenate

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ABSTRACT

Two high-performance liquid chromatographic techniques for the analysis of phosphocitrate, a metabolite synthesized by the cell that is thought to play a role in a number of physiological as well as pathological events, have been developed. The mass spectra of the isolated compound obtained under fast-atom bombardment and collisionally activated decomposition mass-analysed ion kinetic energy conditions are also reported.

INTRODUCTION

Phosphocitrate (PC), first detected in liver mitochondria and in biological fluids, such as urine, bile and saliva, has been reported to be the most powerful inhibitor of calcium phosphate crystallization [1,2]. In mitochondria, PC is postulated to control the level and the nature of calcium accumulation, thus limiting serious damage to cells or to other subcellular structures through uncontrolled metabolism.

Its potential in preventing the events leading to metastatic calcifications had already been established in animal models mimicking different human diseases. In rats, for example, PC may prevent the uptake and crystallization of calcium phosphate in mitochondria and cytosol of renal tubular cells [3], as well as the formation of kidney stones [4]. It was also observed that the compound has the capacity to prevent uremia in animals affected by renal failure caused by an excess of phosphate in the diet [5]. Moreover, PC inhibits aortic calcification, thus interfering in one of the steps of the atherogenetic process [6], and restricts the uptake of calcium and the low-density lipoproteins into smooth muscle cells [7], which is recognized to be another important phase in the overall development of the same process [8,9].

Evidence has recently been obtained for an enzymic synthesis of PC, through the identification of the reaction product by means of enzymic digestion of assay mixtures, high-performance

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liquid chromatography (HPLC) and ^1H NMR spectroscopy [10].

In the course of experiments performed to establish whether a citrate kinase activity would be present in human kidneys, as had been observed in rat liver [10], we followed the formation of PC by HPLC separation of the reaction mixture and its further characterization by mass spectrometry (MS).

This paper describes two HPLC techniques for the identification of the compound and, for the first time, the mass spectra of PC obtained under fast-atom bombardment (FAB) conditions. The primary fragmentation patterns, determined by collisionally activated decomposition (CAD) mass-analysed ion kinetic energy (MIKE) spectra, of molecular species of the chemically and enzymically synthesized PC are also provided.

EXPERIMENTAL

Chemicals and reagents

All chemicals and reagents were of analytical grade. Potassium dihydrogenphosphate, phosphoric acid, hydrochloric acid, sodium hydroxide, sodium citrate, sodium chloride, magnesium chloride and Tris were purchased from Carlo Erba (Milan, Italy). Glycerol, mannitol, sucrose, ATP and N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid) (HEPES) were obtained from Sigma (St. Louis, MO, USA).

Water was doubly distilled and purified using a Milli-Q system (18 MΩ/cm resistivity).

PC standards were prepared as reported previously [2,11] and generously provided by Dr. Sallis (Biochemistry Department, University of Tasmania, Hobart, Australia).

Human kidney homogenate

A 10% human kidney homogenate was prepared essentially according to Schnaitman and Greenawalt [12], in 225 m*M* mannitol, 75 m*M* sucrose and 1 m*M* HEPES (pH 7.4).

Enzymic reaction

A typical microassay system had a volume of 100 μl, and contained 280 μg of proteins, 1 m*M* ATP, 15 m*M* magnesium chloride and 20 m*M* sodium citrate in a 40 m*M* Tris–HCl buffer (pH 7.6). The incubation time was 60 min at 37°C, and the reaction was stopped by adding 5 μl of 16 *M* phosphoric acid. After centrifugation of the mixture at 6000 *g* for 2 min, a 20-μl aliquot was analysed by HPLC.

HPLC apparatus

A Beckman system (GOLD Model) (San Ramon, CA, USA), equipped with a UV–visible detector, was used. Monitoring at two wavelengths (220 and 260 nm) permitted detection of all relevant compounds.

HPLC separation

All the solvents used as chromatographic eluents were filtered through 0.22-μm filters (GVWP 047 00, Millipore, Bedford, MA, USA) and degassed by sonication.

Reversed-phase HPLC was performed using a Beckman Ultrasphere ODS (5 μm, 80 Å, 250 mm × 4.6 mm I.D.) C_{18} column, equilibrated for 120 h with 10 m*M* potassium dihydrogenphosphate solution (adjusted to pH 2.0 with phosphoric acid) containing 5 m*M* tetrabutylammonium sulphate. The mobile phase was 10 m*M* potassium dihydrogenphosphate, adjusted with phosphoric acid to pH 2.0, used under isocratic conditions.

Polar bonded-phase chromatography was performed using a Beckman Ultrasil (10 μm, 80 Å, 250 mm × 4.6 mm I.D.) NH_2 column. The mobile phase was buffer A (10 m*M* potassium dihydrogenphosphate adjusted with phosphoric acid to pH 2.2) and buffer B (buffer A plus 300 m*M* sodium chloride); 100% buffer B was reached with a linear gradient in 20 min.

In both cases the flow-rate was 1.0 ml/min.

MS apparatus and conditions

All MS measurements were performed with a double-focusing, reversed-geometry VG ZAB2F (VG Analytical, Manchester, UK) instrument operating in FAB conditions (8 keV, xenon atoms bombarding glycerol solutions of the sample). CADs were detected by MIKE spectrometry [13]. The helium pressure in the collision cell

placed in the second field-free region was such as to reduce the main beam intensity to 40% of its usual value.

RESULTS AND DISCUSSION

Because PC seems able to interfere with the pathogenesis of the kidney diseases related to calcium accumulation [3–5], experiments were performed to detect citrate kinase activity in human kidney homogenates. Such an enzyme would ensure the cell's capacity to synthesize PC, thus protecting it from the damage derived from accumulation of calcium in a crystalline form [2].

As already reported [10], the problem was first approached by running the assay mixture containing the human kidney homogenate on an HPLC reversed-phase column. As shown in Fig. 1a, the PC standard, chemically synthesized as described by Williams and Sallis [11], elutes at the same retention time as a peak observed in the assay mixture (Fig. 1b). These results are consistent with those already published, dealing with the biological synthesis of PC [10] when a rat liver homogenate was used as a source of enzymic activity.

In order to have further evidence on the nature of the peak that elutes with the standard, the peaks of both the chemically and enzymically synthesized PC were collected and submitted to MS analysis.

Fig. 2a and b show the partial FAB mass spectrum of both the peak of the standard and that of the enzymic assay collected from the column (as

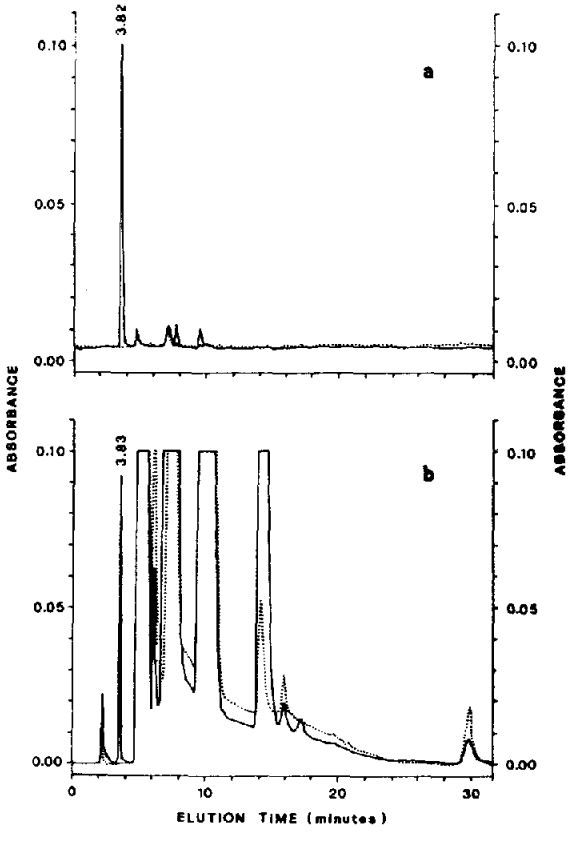

Fig. 1. HPLC elution profiles, obtained with the reversed-phase column, of (a) standard PC and (b) the enzymic reaction mixture (see Experimental). Detection wavelength: (———) 220 nm; (· · ·) 260 nm.

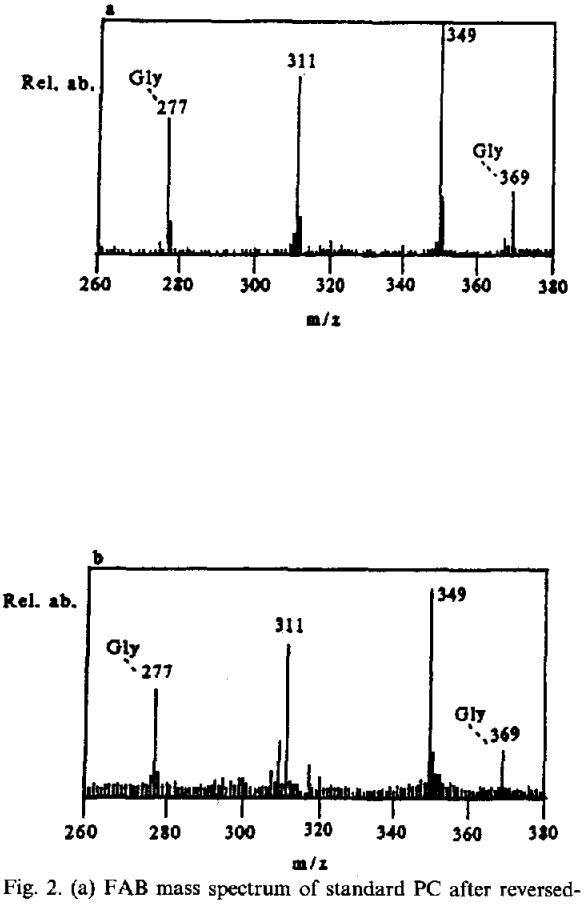

Fig. 2. (a) FAB mass spectrum of standard PC after reversed-phase HPLC. (b) FAB mass spectrum of enzymically synthesized PC after reversed-phase HPLC. Rel. ab. = relative abundance; Gly = glycerol.

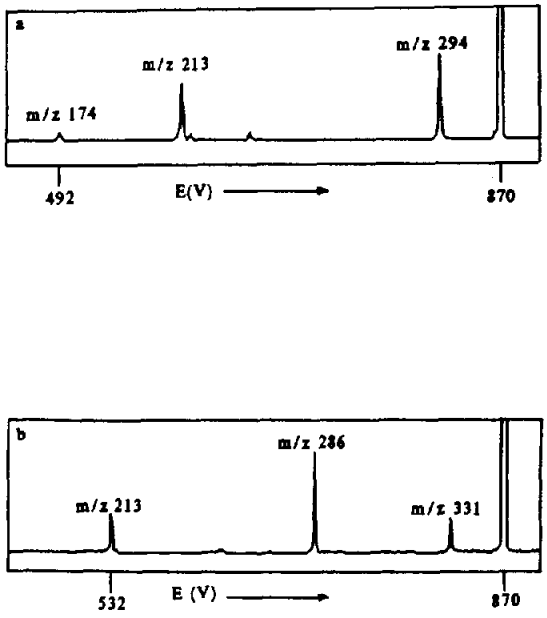

Fig. 3. MIKE spectra of the ions at m/z 311 (a) and 349 (b) present in the FAB spectra of Fig. 2.

in Fig. 1a and b). Both spectra show abundant ions at m/z 311 and m/z 349 with a signal-to-noise ratio higher than 10.

FAB-MS is known to be highly effective in the identification of the molecular masses of polar substances, owing to the formation of protonated or cationized molecular species. In contrast, it is poorly effective for structural identification, owing to the low-energy deposition and the consequent scarcity of fragment ions. In order to gain such structural information, some collisional experiments were carried out, by means of MIKE spectrometry, performed on the two relevant ions at m/z 311 and 349 (see Fig. 3). The related fragmentation patterns are reported in Fig. 4. As may be inferred, both molecular species at m/z 311 and 349 show a primary loss of water, responsible for the formation of ions at m/z 293 and 331, respectively. However, only the ionic species at m/z 331 further decomposes through decarboxy-

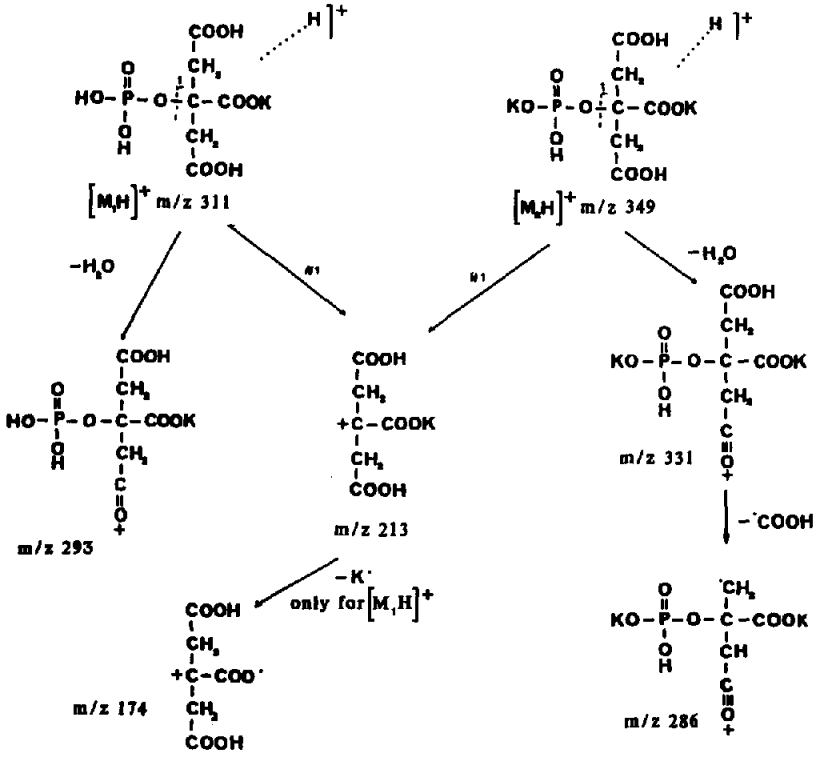

Fig. 4. Fragmentation patterns of the two molecular species of PC at m/z 311 ($[M_1H]^+$) and m/z 349 ($[M_2H]^+$).

lation, giving rise to an ion at m/z 286. An interesting decomposition is that originating from cleavage 1 (Fig. 4), common to both parent ions at m/z 349 and 311, leading to the common fragment at m/z 213. These data for ionic species at m/z 311 and 349 are in agreement with the structures of the mono- and dipotassium salts, and the MS fragmentation patterns indicate that for the species at m/z 349, one of the potassium atoms must be contained in the phosphate moiety of the molecule.

In principle, the presence of potassium atoms could be ascribed to K^+ addiction occurring during FAB. However, variation of the FAB matrix acidity suggested that potassium atoms probably arise from the original molecule under these chromatographic conditions.

From these results, it appears that enzymic ac-

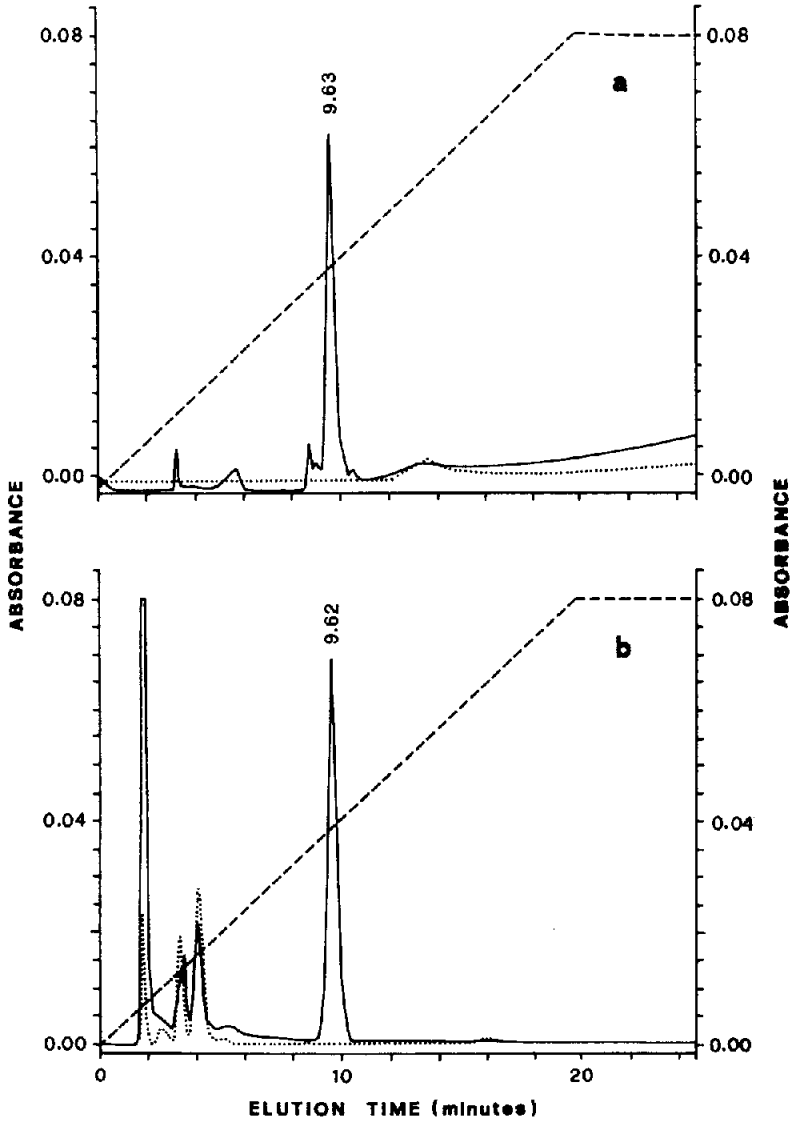

Fig. 5. HPLC elution profiles, obtained with the polar bonded phase Ultrasil column, of (a) standard PC and (b) the enzymic reaction mixture. The dashed line shows the linear gradient of buffer B (buffer A plus 300 mM sodium chloride) (see Experimental). Detection wavelength: (———) 220 nm; (· · ·) 260 nm.

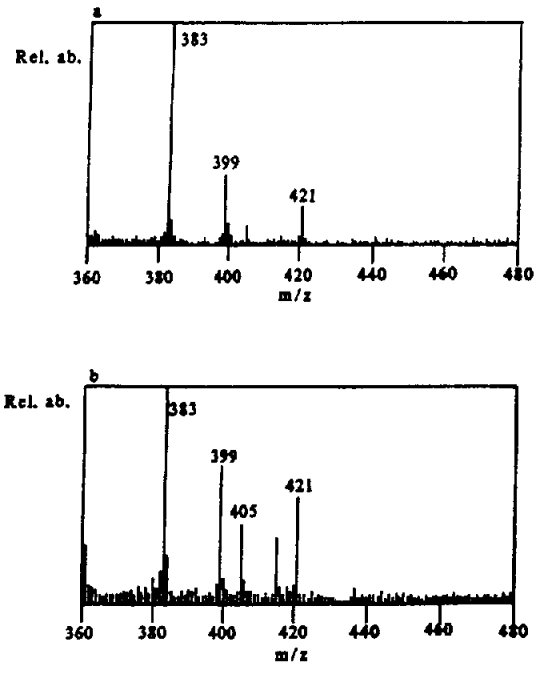

Fig. 6. (a) FAB mass spectrum of standard PC after polar bonded phase HPLC. (b) FAB mass spectrum of enzymically synthesized PC after polar bonded phase HPLC.

tivity capable of synthesizing PC also occurs in human kidneys. In fact, FAB-MS and the fragmentation patterns lead to an unequivocal structural identification of PC formed in the assay mixture.

However, the need for a more suitable and reproducible chromatographic method led us to the use of a weak anion-exchange column. The corresponding elution patterns of the PC standard and the assay mixture are shown in Fig. 5a and b. The PC standard elutes as a single peak at 9.63 min, and the assay mixture shows a peak with the same retention time. To obtain further evidence on the nature of this peak, both the standard PC and the corresponding peak in the sample were analysed by MS. Again, the peak of the standard and the peak of the assay mixture eluting at 9.62 min give practically identical FAB mass spectra (see Fig. 6), showing an intense peak at m/z 383 and less abundant ions at m/z 399 and 421. The fragmentation pattern of the ion at m/z 383, as obtained by collisional experiments under MIKE conditions (see Figs. 7 and 8), shows the formation of intense ions at m/z 360, 316, 293, 257 and 141. Such data are in agreement with the sodium salification of all the acid hydrogens present in the molecule: as shown in Fig. 8, the ion at m/z 141 corresponds to sodium phosphate originating from the cleavage of a carbon–oxygen bond; the ion at m/z 360 is due to a primary sodium loss, and the ion at m/z 316 originates from a loss of a COONa radical. Finally, the cleavage of the P–O bond is responsible for the formation of the peak at m/z 257. The ion at m/z 399 corresponds to partial potassium salification of PC. Its molecular mass results from the presence of four sodium and one potassium ion in the $[MH]^+$ species of PC, and the ion at m/z 421 can be attributed to the sodium salt of same species.

The extensive salification cannot be ascribed to the FAB conditions adopted; this is not surprising if one considers that the chromatographic technique employed with this type of column uses a NaCl gradient (see Fig. 5a and b) to separate the compounds.

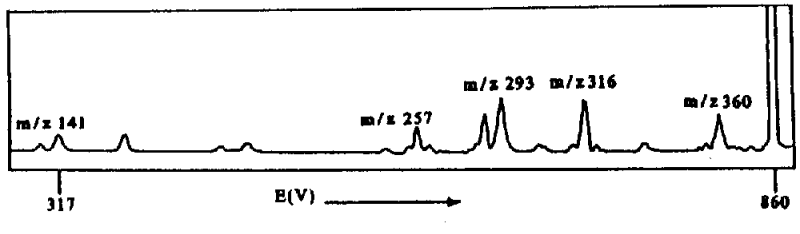

Fig. 7. MIKE spectrum of the molecular species at m/z 383 present in the FAB spectra of Fig. 6.

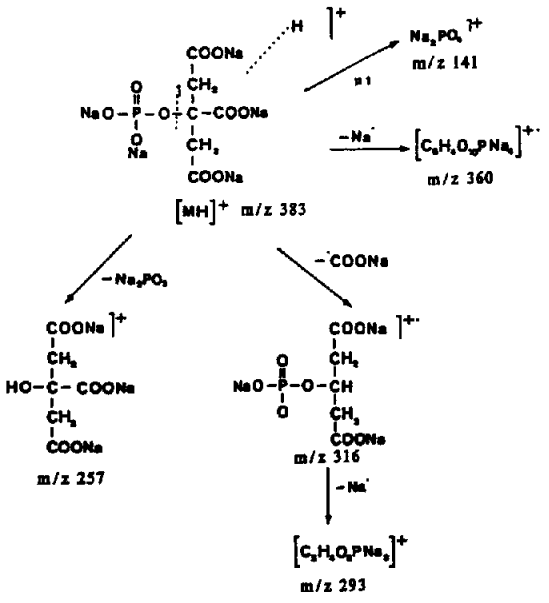

Fig. 8. Fragmentation pattern of the molecular species at m/z 383 ($[MH]^+$).

CONCLUSION

The results presented in this paper demonstrate that PC can be studied by FAB-MS. When using this technique, one has to be aware that the ionic species arising from the FAB and CAD-MIKE analysis could be different, depending on the chromatographic matrices employed for the separation of the molecules. Moreover, these data prove that citrate kinase is present in human kidneys, and this finding will certainly contribute to a better understanding of the pathogenesis of kidney diseases related to calcium accumulation.

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