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Ion-pair high-performance liquid chromatography of purine compounds in the small intestinal mucosa of children with coeliac disease

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ABSTRACT

Isocratic methods are described for the separation of purine compounds in specimens of jejunal mucosa available by peroral biopsy from patients with coeliac disease. The first mode provided separation of both the ionic nucleoside monophosphates and the non-ionic nitrogen bases and nucleosides. The second mode was applicable to analyses of the nucleoside mono-, di- and triphosphates only. These chromatographic procedures were carried out with tetrabutylammonium phosphate as an ion-pair modifier and different concentrations of acetonitrile in the mobile phase. The modes are flexible in that they permit the optimization of the separation of these compounds according to the purpose of the investigation. The results of studies on purine metabolite patterns in coeliac mucosa are discussed.

INTRODUCTION

Recently we have developed ion-pair reversed-phase chromatographic methods [1,2] which provided the possibility of the gradient separation and determination of purine and pyrimidine metabolites in different cells and tissue specimens. Nevertheless, sometimes the clinical and experimental reality necessitates the employment of simpler isocratic chromatographic procedures for the separation of nucleic acid constituents. However, only gastrointestinal mucosa from patients with malignant cancer has been investigated to determine the level of nucleosides and bases by high-performance liquid chromatography (HPLC) [3].

As the synthesis of nucleic acid constituents is related directly to cell division, it was postulated that the activities of these enzymes could be correlated with changes in cellular proliferation and migration rates in the intestine [4]. However, the *in vivo* activity of the purine and pyrimidine enzymes depends not only on the capacity of the enzymes but also on the concentration of the substrates of the metabolic pathways. Therefore, differences in the profiles of nucleotides, nucleosides and bases in normal and coeliac mucosa could provide important information on the nucleic acid metabolism in mucosa of patients with coeliac disease.

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EXPERIMENTAL

Chemicals

Nucleic bases, nucleosides and their mono-, di- and triphosphates were purchased from Sigma (St. Louis, MO, U.S.A.), potassium dihydrogenphosphate and ammonium dihydrogenphosphate from Prolabo (Paris, France), tetrabutylammonium phosphate (TBA) from Beckman (San Ramon, CA, U.S.A.), triethanolamine hydrochloride from ICN Pharmaceuticals (Cleveland, OH, U.S.A.) and HPLC-grade acetonitrile (CromAR) from Promochem (Wesel, F.R.G.). Reverse-osmosis water from a Milli-R/Q water purifier (Millipore, Bedford, MA, U.S.A.) was passed through a Norganic cartridge (Waters Assoc., Milford, MA, U.S.A.).

Instrumentation

The HPLC analysis was carried out using a GOLD Method Development System, consisting of a model 126 programmable solvent module, a model 167 scanning detector module and an Altex 210A injection valve. GOLD solftware version 3.1 was used for data collection and controller functions. An Ultrasphere XL C_{18} (3-μm) cartridge column (75 mm × 4.6 mm I.D.) and TBA as ion-pair reagent were used in both separation modes. Standards were prepared at concentrations of about 20–40 μM. The injection volume of both the standard mixture and biological samples was 50 μl. Concentration measurements were performed using the external standard method. Peaks were identified from their retention times and absorbance ratios at 254 and 280 nm.

Sample preparation

Human intestinal mucosa was obtained by peroral jejunal biopsy. Each biopsy specimen was weighed (2–5 mg), immediately placed in ice-cold saline and homogenized in 200 μl of cold 0.6 M perchloric acid using a micro Waring blender. The mixture was then centrifuged at 3000 g for 5 min and the supernatant was neutralized with 40 μl of 1.2 M potassium carbonate–0.6 M triethanolamine. The samples were centrifuged to remove precipitable perchlorate and the resulting supernatants were stored at −40°C until HPLC analysis.

RESULTS

The first isocratic method was developed for the separation of adenosine and guanosine mono-, di- and triphosphates. The method was based on the different number of phosphate moieties and consequently different charges of nucleotides under the conditions of the ion-exchange retention mechanism. Separations were performed with 10 mM $NH_4H_2PO_4$–2 mM TBA–20% acetonitrile (pH 6.3) as the mobile phase and the flow-rate was initially 0.5 ml/min and was changed to 1 ml/min after 7 min. The application of a XL C_{18} cartridge column with a 3-μm particle size allowed the flow-rate to be increased to 1.0 ml/min before the appearance of the first triphosphate peak (GTP) and provided for a reasonable pressure range (up to 2000 p.s.i.). The use of the flow-rate step gradient, a low ionic strength and a high content of organic modifier under saturation conditions of TBA on a C_{18} stationary phase ensured good resolution of purine nucleoside mono-, di- and triphosphates (Figs. 1 and 2).

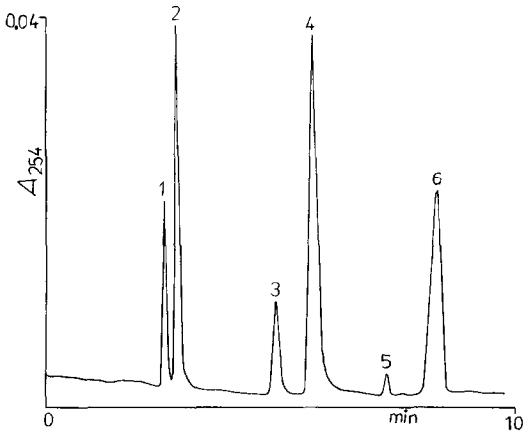

Fig. 1. Ion-pair reversed-phase separation of a standard mixture under isocratic conditions with a flow-rate step gradient. Column, Ultrasphere-XL C_{18} cartridge (75 mm × 4.6 mm I.D.); mobile phase, 10 m*M* $NH_4H_2PO_4$–2 m*M* TBA–20% acetonitrile (pH 6.3); initial flow-rate, 0.5 ml/min, increased to 1.0 ml/min after 7 min. Peaks: 1 = GMP; 2 = AMP; 3 = ADP; 4 = GDP; 5 = GTP; 6 = ATP.

The second isocratic method was developed for the simultaneous analysis of neutral bases and nucleosides and their charged monophosphates. Separations were performed with 40 m*M* KH_2PO_4–1 m*M* TBA–2% acetonitrile (pH 5.1) as the mobile phase at a flow-rate of 0.5 ml/min. The conditions were slightly changed from these reported previously [2] and adapted to the Ultrasphere C_{18} column. The use of a medium ionic strength, a low content of organic modifier under non-saturation conditions of the ion-pair reagent on the stationary C_{18} phase provided conditions under which the peaks of charged nucleoside monophosphates appeared at the end of the chromatogram, while the ordinary pattern of separation of bases and nucleosides remained unchanged (Fig. 3).

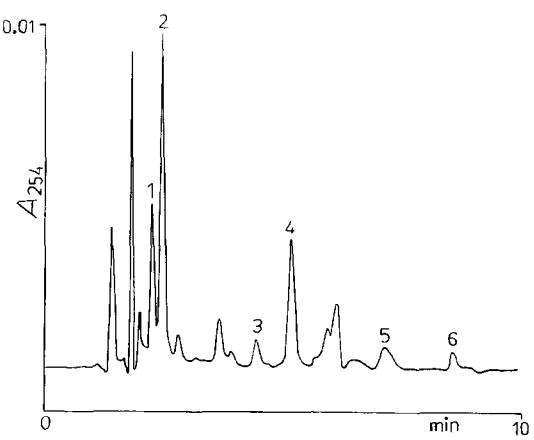

Fig. 2. Separation of adenine and guanine nucleotides in coeliac mucosa. Conditions and peaks as in Fig. 1.

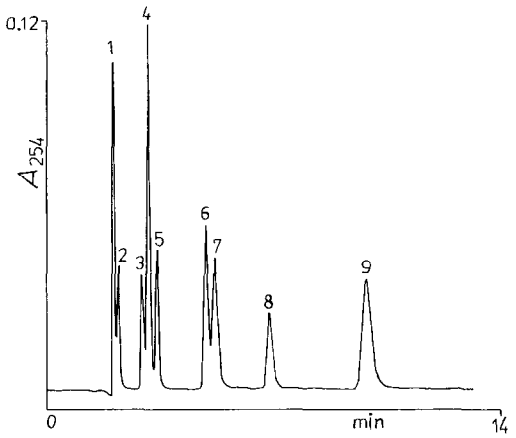

Fig. 3. Ion-pair reversed-phase separation of purine nucleic bases, nucleosides and their monophosphates under isocratic conditions. Column, Ultrasphere-XL C_{18} cartridge (75 mm × 4.6 mm I.D.); mobile phase, 40 m*M* KH_2PO_4–1 m*M* TBA–2% acetonitrile (pH 5.1); flow-rate, 0.5 ml/min. Peaks: 1 = hypoxanthine; 2 = xanthine; 3 = adenine; 4 = inosine; 5 = guanosine; 6 = GMP; 7 = IMP; 8 = adenosine; 9 = AMP.

DISCUSSION

The small intestinal mucosa is one of the most actively regenerating mammalian tissues. In gluten-sensitive coeliac disease the intestinal mucosa shows marked structural abnormalities with flattening and a loss of villi. Simultaneously, the increase in the depth of the intestinal crypts is associated with the number of cells in mitosis in the crypts and a widening of the mitotic zone [5]. However, little is known about biochemical abnormalities in coeliac mucosa.

Table I demonstrates the existence of certain patterns in the purine metabolic pools in the normal and coeliac mucosa. It was found that the levels of ADP and particularly of ATP markedly increased in coeliac mucosa, with no apparent changes in the pool of AMP. A similar pattern was observed with the guanine nucleotides.

The concentration of IMP in coeliac mucosa was 50% of that in normal mucosa. The concentrations of adenosine and adenine changed relatively little. In contrast, in subtotal villous atrophy, the sharp increase in the xanthine level and the slight increase in the uric acid concentration are attributed to the high activity of the enzyme xanthine oxidase present in the mucosa [6]. The increase in the inosine concentration with decrease in the IMP level indicates the functioning of IMP nucleotidase in coeliac mucosa.

As intestinal mucosa has a limited capacity for *de novo* synthesis of purine nucleotides [7], this tissue is dependent on salvage pathway activity for the provision of purine nucleotides. Apparently, the accumulations of ATP and GTP may be ascribed to the very high activity and amount of adenylate and guanylate kinases in coeliac mucosa and is directly related to the marked increase in the rate of cellular profileration. On the other hand, the high level of the end products of purine catabolism in coeliac mucosa may be correlated with the changes in the cellular turnover.

Future investigations will be aimed at understanding the alterations in purine

TABLE I

CONTENT OF PURINE COMPOUNDS IN NORMAL AND COELIAC INTESTINAL MUCOSA DETERMINED BY HPLC

Metabolite	Concentration (nmol/g wet weight, mean ± S.E.M.[a])	
	Normal mucosa ($n=5$)	Coeliac mucosa ($n=6$)
ATP	180 ± 62	950 ± 204
ADP	630 ± 93	796 ± 104
AMP	882 ± 44	795 ± 68
GTP	267 ± 64	620 ± 120
GDP	78 ± 10	270 ± 74
GMP	354 ± 49	345 ± 48
IMP	316 ± 102	142 ± 22
Ado	101 ± 15	90 ± 16
Ade	48 ± 11	36 ± 8
Ino	61 ± 12	79 ± 10
Hyp	60 ± 10	42 ± 8
Xan	14 ± 1	43 ± 7
Uric acid	75 ± 13	80 ± 24

[a] Standard error of the mean

metabolism in patients with coeliac disease in remission who have been on a gluten-free diet.

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