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Journal of Chromatography A, 732 (1996) 271–275

JOURNAL OF CHROMATOGRAPHY A

Separation of twenty isomers of ribonucleotides and deoxyribonucleotides by reversed-phase ion-pairing high-performance liquid chromatography

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First received 1 November 1994; revised 13 November 1995; accepted 13 November 1995

Abstract

A procedure is described for the separation of 20 isomers of ribonucleotides and deoxyribonucleotides by reversed-phase ion-pairing high-performance liquid chromatography using a Waters μBondapak C_{18} 10 μm column and a gradient elution system consisting of phosphate buffer (0.05 *M*, pH 5.45) and methanol. The method is relatively fast, taking 40 min for each run, and quantitative. Nucleosides and nucleobases were eluted earlier in the system and did not interfere with the separation of nucleotides. Sample preparation is simple and prior group separation is not necessary. The method has been used successfully for the identification and quantification of nucleotides produced from nucleic acid degradation during yeast autolysis.

Keywords: *Saccharomyces cerevisiae*; Ribonucleotides; Deoxyribonucleotides; Nucleotides

1. Introduction

Nucleotides, particularly 5′-nucleotides such as 5′-GMP, are important flavouring agents found in many foods and beverages [1]. These compounds are also formed from the degradation of DNA and RNA during the autolysis of yeasts [2]. Yeast autolysates containing these products have commercial applications in the food industry as flavouring ingredients [3], while yeast autolysis and the degradation of nucleic acids during wine, champagne and beer fermentations can impact on the sensory quality of these beverages [4,5]. Yeast cells contain a range of different nucleases [6] and, consequently, autolytic degradation of nucleic acids is expected to yield different isomers of nucleotides as well as nucleosides and nucleobases. To study the profiles of degradation products of nucleic acids, methods that separate and quantify a complex mixture of 5′-, 3′- and 2′-isomers of nucleotides, nucleosides and nucleobases become a prerequisite.

Many high-performance liquid chromatography (HPLC) procedures have been developed for separating nucleotides and their isomers and the methodology has been reviewed [7–9]. Nucleotides are negatively charged at pH less than 7.0 so that anion exchange is the mode of HPLC most commonly used for their separation [8,9]. Reversed-phase chromatography has been

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SSDI 0021-9673(95)01264-8

used to overcome the problems of baseline drifting and long running time, which are commonly encountered in ion-exchange HPLC [10–12]. However, reversed-phase systems give poor retention and resolution of charged molecules and, therefore, not all nucleotides can be resolved by this mode of chromatography. Reversed-phase ion-pairing chromatography, in which an ion-pairing reagent is included in the mobile phase, appears to offer better retention and resolution than reversed-phase chromatography and more stable baseline than ion-exchange chromatography. Methods based upon this mode of HPLC give good resolution of nucleotides [13–15]. However, these methods have not separated all the isomers of nucleotides that are formed from the degradation of DNA and RNA.

In a previous paper [16], we described a reversed-phase HPLC procedure for the separation of five nucleobases, eight ribo- and deoxyribonucleosides and eight 5′-ribo- and deoxyribonucleotides. However, this procedure did not resolve the different isomers of nucleotides.

We report, here, a reversed-phase ion-pairing HPLC procedure which can separate a mixture of all 20 isomers (5′- 3′- and 2′-) of the ribonucleotides and deoxyribonucleotides of which RNA and DNA are composed. Nucleosides and bases were eluted early and did not interfere with the separation of nucleotides. The analysis is relatively rapid, taking 40 min for one run. It has been applied to the identification and quantification of DNA and RNA degradation products in yeast autolysates.

2. Experimental

2.1. Instrumentation

HPLC was performed with a Waters liquid chromatography system (Millipore, Milford, MA, USA) consisting of two Model 510 pumps, a Model 660 gradient controller, a U6K sample injector and a Model 440 absorbance detector operating at two wavelengths of 245 and 280 nm. Retention times and peak areas were recorded with a Waters Model 745 data module.

2.2. Column

A Waters μBondapak C_{18} 10 μm stainless-steel column (30 cm × 3.9 mm I.D) was used. The column packing was composed of silica-based 10 μm irregular beads bonded with 10% load of C_{18} material and was protected by a Waters guard column made of the same packing material.

2.3. Chemicals and chromatographic standards

Standards of individual nucleotides, nucleosides and purine and pyrimidine bases were purchased from United States Biochemical Corp. (Cleveland, OH, USA). The ion-pairing reagent, PIC A (tetrabutylammonium phosphate), was obtained from Waters (Millipore, Sydney, Australia). All other chemicals and solvents were analytical grade or specially purified for HPLC work.

2.4. Chromatographic conditions

Elution was performed with a gradient system comprised of two eluents. Eluent A was a solution of 0.05 *M* KH_2PO_4 and 0.005 *M* Waters PIC A (ion-pairing reagent) adjusted to pH 5.45 ± 0.02 with 2 *M* phosphoric acid. Eluent B was 100% methanol (HPLC grade). Prior to use, all eluents were filtered through a membrane filter, pore size 0.45 μm (Millipore, Sydney, Australia), and degassed by vacuum and sonication for 5 min.

Before injection of a sample, the column was equilibrated with eluent A for 10 min to stabilise the baseline. After injection, elution was started with 100% eluent A and 0% eluent B. The ratio of eluent B in the system was increased linearly from 0 to 10% over 10 min, and the elution was maintained at this condition (90% A, 10% B) until the run was completed (30 min). The system was then returned to its starting condition (100% A) for the next run. One analysis took 40 min with the flow-rate of eluent being main-

tained at 1.5 ml/min throughout the run. Elution was performed at ambient temperature (approximately 25°C.

2.5. Preparation of autolysate samples for HPLC analysis

Cells of *Saccharomyces cerevisiae* x2180 were grown in 0.5% yeast extract–5% glucose medium at 25°C for 48 h. Cells were harvested by centrifugation, washed, and suspended in 0.2 *M* sodium phosphate–citric acid buffer, pH 7.0. Autolysis was initiated by incubating the suspension at 40°C with orbital shaking at 200 rpm. Samples were withdrawn daily and separated by centrifugation (5000 *g* for 5 min) into cell pellet and cell-free autolysate fractions. The autolysate fraction was filtered through a 0.45-μm membrane and analysed by HPLC without further treatment.

2.6. Recovery of nucleotides

The recovery of nucleotides during HPLC analysis of yeast autolysates was examined by spiking standard 3′-AMP (100 μg/ml) into autolysate samples and then separately injecting the standard, the autolysate and the spiked autolysate sample. This test was performed three times. Recovery was calculated as the peak area of 3′-AMP of the spiked sample divided by the sum of the peak areas of standard 3′-AMP and that of the autolysate sample.

3. Results and discussion

Fig. 1 shows the separation of a standard mixture of twelve 5′-, 3′- and 2′-isomers of ribonucleotides and eight 5′- and 3′-isomers of deoxyribonucleotides by the HPLC system. Peaks of the chromatogram were identified by matching their respective retention times with those of standard compounds injected individually. Eighteen of the 20 components were separated, but 5′-dGMP and 5′-dTMP did not separate and eluted as one peak (peak number 8). All the components were eluted within 30 min.

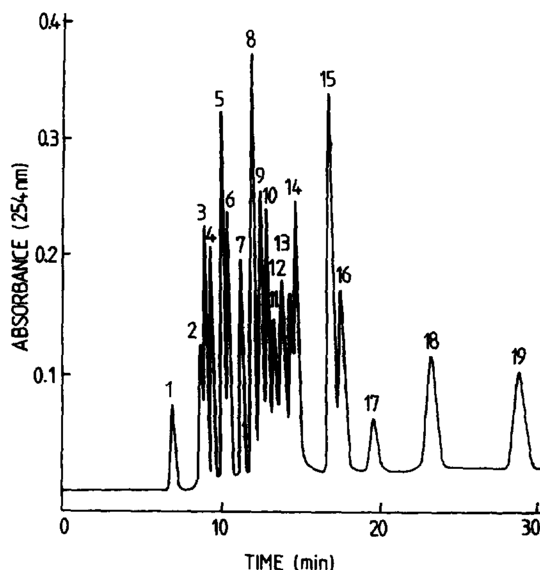

Fig. 1. Chromatogram of a standard mixture of nucleotides (100 μg/ml each). Column, Waters μBondapak C_{18} 10 μm; eluent A, 0.05 *M* KH_2PO_4 and 5 m*M* PIC A ion-pairing reagent (pH 5.45); eluent B, methanol. Elution program: ratio of eluent B was increased linearly from 0 to 10% over 10 min and held at this condition until the run was finished. Injection volume, 20 μl; detection wavelength, 254 nm; t_0 = 1.19 min where t_0 is the retention time of a non-retaining compound (calculated as system void volume/flow-rate); the void volume was determined with sodium nitrite (10 m*M*). Peaks: 1 = 5′-CMP; 2 = 5′-dCMP; 3 = 5′-UMP; 4 = 3′-dCMP; 5 = 5′-GMP; 6 = 2′-CMP; 7 = 3′-CMP; 8 = 5′-dGMP and 5′-dTMP; 9 = 3′-dGMP; 10 = 2′-UMP; 11 = 3′-UMP; 12 = 5′-AMP; 13 = 3′-dTMP; 14 = 3′-GMP; 15 = 2′-GMP; 16 = 5′-dAMP; 17 = 3′-dAMP; 18 = 3′-AMP; 19 = 2′-AMP.

After the system was returned to its starting conditions (100% A), an equilibration time of 10 min was needed to stabilise the baseline before the next injection. Therefore, one chromatographic run took 40 min to complete.

It was critical to maintain the pH of eluent A at 5.45 ± 0.05 in order to achieve acceptable resolution. Deviation from this pH decreased resolution, with resultant overlapping of some peaks. Decreasing pH of eluent A in 0.25 intervals from 5.45 to 3.50 tended to improve resolution of early peaks, but the improvement was offset by a greater loss of resolution for later peaks. As noted already, the system conducted at pH 5.45 did not resolve 5′-dGMP and 5′-dTMP (peak 8, Fig. 1). These two components were

resolved by decreasing the pH of eluent A to 4.85 (5′-dGMP elution time 11.85 min; 5′-dTMP elution time 12.62 min), but resolution of several other components was lost. Increasing the pH of eluent A in 0.25 intervals from 5.45 to 6.5 caused overlapping of 3′-UMP and 5′-AMP without improving resolution of other peaks.

Decreasing the ratio of methanol (eluent B) in the elution system from 10 to 8% increased retention of the peaks, but failed to improve peak resolution. Increased retention without improving resolution was also observed when the gradient for increasing eluent B from 0 to 10% was prolonged from 10 to 15 min. Increasing the ratio of methanol in the elution system (up to 15%), or shortening the time for eluent B to increase from 0 to 10% from 10 to 5 min gave decreased peak retention and resolution.

Fig. 2 shows the resolution of nucleic acid degradation products in an autolysate of *S. cerevisiae* when analysed by the HPLC procedure. Most of the peaks in the chromatogram were identified by comparing their retention times with those of the standards. The peaks eluting before 6 min were nucleosides and bases which eluted earlier than nucleotides in this system. Nucleosides and nucleobases formed from the degradation of RNA and DNA were not well resolved by this system. Separation of these components along with eight 5′-nucleotides was accomplished by a reversed-phase HPLC procedure described previously [16]. Some of the peaks in the chromatogram were not identified. They are probably di- or trinucleotides as these compounds are also expected to be present in yeast autolysates [17].

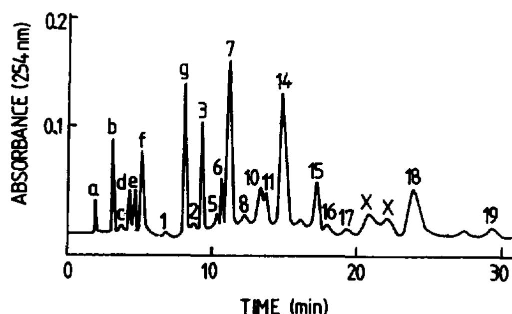

Fig. 2. Chromatogram of a sample of *Saccharomyces cerevisiae* autolysate; the HPLC system has been described in Fig. 1. The autolysate was taken after cells had been autolysed for 8 days at 40°C and pH 7.0. Peaks: a–g = Nucleosides and bases; X = unidentified peaks; for identification of numbered peaks, refer to Fig. 1.

Solutions of individual standard compounds at different concentrations were injected onto the HPLC system, and standard curves were obtained by plotting the peak areas against concentrations of the standards. Good linearity was demonstrated for all compounds, with coefficients of determination (r^2) being greater than 0.98.

To test the efficiency of recovery of nucleotides by the HPLC procedure, yeast autolysate samples were spiked with 100 μg/ml of standard 3′-AMP and the standard, the autolysate, and the spiked autolysate sample were injected into the system separately. The recovery of 3′-AMP was 96.8, 97.9 and 97.0% for three separate trials.

Edelson et al. [18] reported an ion-exchange HPLC procedure that separated 2′-, 3′- and 5′-nucleotides and cyclic ribonucleotides of adenosine, cytidine and guanosine. However, the nucleotides were not separated in one single chromatographic run and the procedure did not separate these components from deoxyribonucleotides and isomers of uridine ribonucleotides. Qureshi et al. [19] resolved 14 isomers of ribonucleotides and two isomers of deoxyribonucleotides (5′- and 3′-dTMP) by ion-pairing HPLC using a mixture of 87.5% water, 10% methanol, 1.5% acetic acid and 1% PIC A reagent as the sole eluent, but this system did not separate these components from the other six isomers of deoxyribonucleotides. Our attempts to modify this procedure by decreasing the ratio of methanol in the eluent from 10 to 8% improved peak resolution, with resultant separation of 16 components out of 20 isomers of ribo- and deoxy-ribonucleotides (data not shown). Further decreases in the ratio of methanol in the eluent gave increases in peak retention without improving resolution, while increases in the ratio of methanol caused loss of peak resolution. Attempts to use a gradient elution system, in which the ratio of methanol was increased linearly from

0 to 8% in various times, improved resolution of the five isomers of cytidine ribo- and deoxyribonucleotide (which were the first five peaks eluted in the system), but failed to improve overall peak resolution.

The reversed-phase ion-pairing HPLC procedure described in this paper is a new method which is able to separate the 20 isomers of the ribonucleotides and deoxyribonucleotides composing RNA and DNA. It is superior to the published methods in that it can resolve more components of nucleotides in a single chromatographic run. A further advantage of the method is that nucleosides and bases are eluted early in the system and do not interfere with the measurements of nucleotides. Therefore, prior group separation is not necessary in analysing samples containing these compounds. The method has been used successfully in identification and quantification of isomers of nucleotides formed from nucleic acid degradation during yeast autolysis and may be used in analysing yeast autolytic products in commodities such as beer, wine and champagne where yeast autolysis is known to occur [4,5].

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