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Short Communication

Separation of nucleotides in homogenates of octopus retina by ion-pair reversed-phase liquid chromatography and identification by mass spectrometry

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ABSTRACT

An isocratic ion-pair reversed-phase liquid chromatographic method has been developed for the determination of thirteen nucleotides including cyclic AMP and cyclic GMP. The resolution capability of this method was evaluated successfully using homogenates of octopus retina, the aim being to elucidate the role of nucleotides (particularly ADP and ATP) in the control of oxidative metabolism. To overcome the inherent lack of specificity of ultraviolet detection we used the coupling of liquid chromatography with mass spectrometry, via a thermospray interface, to confirm the identity of the nucleotides of interest in the biological samples.

INTRODUCTION

Analysis of purine and pyrimidine compounds is an essential step in many areas of biomedical research. Liquid chromatographic (LC) techniques have greatly facilitated the analysis of nucleotides, nucleosides and bases in biological materials [1,2]. Nucleotides have usually been separated by ion-exchange chromatography [3–7]. Reversed-phase chromatography, with or without ion-pairing, is increasingly used for separating the phosphate esters [7–15].

This paper describes an ion-pair reversed-phased high-performance liquid

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chromatographic (HPLC) method that allows the complete separation of thirteen nucleotides in *ca.* 30 min. The analytical separation is obtained using a 5-μm LiChrosorb RP-18 column at room temperature under isocratic conditions and the quantification by UV spectrophotometry at 254 nm. This method has been tested for separating nucleotides in homogenates of retina from octopus (*Octopus vulgaris*) as part of a long-term project exploring the role of nucleotides (particularly ADP and ATP) in the control of oxidative metabolism [16]. Because nucleotides occur in complex biological matrices, different detection systems are required to permit their unambiguous identification. In addition to HPLC–UV, we coupled LC with mass spectrometry (MS) to identify the structures of the nucleotides [17–19] of interest in octopus retina homogenates.

EXPERIMENTAL

Reagents and chemicals

Nucleotides of the highest purity available were purchased from Sigma (St. Louis, MO, U.S.A.) and NAD from Boehringer (Mannheim, Germany). Tetrabutylammonium hydroxide (40% in water purum grade) was from Fluka (Buchs, Switzerland). Disodium hydrogenphosphate and orthophosphoric acid (85%) were from Merck (Darmstadt, Germany), both of analytical grade. Acetonitrile (HPLC grade) was from Romil Chemicals (Shepshed, U.K.).

Stock solutions of nucleotides (200 μM) were prepared in ultrapure water, using a Milli-Q water purifier from Millipore (Bedford, MA, U.S.A.), and kept frozen at -20°C for up to three months. Standard working solutions were prepared with each series of assays.

Instrumentation

The LC system consisted of a Model M-45 HPLC pump from Waters Assoc. (Milford, MA, U.S.A.), a Rheodyne injector, Type 7025, with a 20-μl loop, a Uvikon LCD 725 UV–VIS detector from Kontron (Zürich, Switzerland) and a 4290 recording integrator from Spectra-Physics (San Jose, CA, U.S.A.). The analytical column (250 mm × 4.6 mm I.D.) was packed with 5-μm LiChrosorb RP-18 and was from Merck.

MS analyses were carried out on a combined liquid chromatograph–mass spectrometer–computer system. An HP 1090 chromatograph was coupled to an HP 5988A mass spectrometer equipped with a thermospray interface. The data system was the computer HP 9000 Serial 310 (2 Mbytes RAM). This apparatus was from Hewlett-Packard (Palo Alto, CA, U.S.A.).

Chromatographic conditions

The mobile phase was prepared by adjusting the pH of a 50 mM solution of disodium hydrogenphosphate to 6.4 by dropwise addition of orthophosphoric acid. This phosphate buffer was mixed with 1 mM of tetrabutylammonium hy-

droxide and 4.8% (v/v) acetonitrile. The mobile phase was prepared in ultrapure water and filtered through a 0.22-μm Millepore filter and degassed just before use. The flow-rate was 1.0 ml/min. The separation was obtained under isocratic conditions at room temperature, and the absorbance of peaks was monitored at 254 nm.

Sample preparation

Pieces of retina were dissected from the eye of octopus under dim red light and perfused in a chamber with oxygenated seawater. After 1 h of dark adaptation the retina was shock-frozen in liquid isopentane and lyophilized for *ca.* 12 h. Samples of lyophilized tissue were weighed on a microbalance and homogenized in iced trichloroacetic acid (5% solution). After 10 min centrifugation at 7128 *g*, the supernatant was collected and washed five or six times with water-saturated diethyl ether until the pH was *ca.* 6. After filtration, the sample was ready to use for LC; 20-μl samples were typically injected.

Calibration and reproducibility

The HPLC–UV method was quantified on the basis of peak areas. Calibration graphs were obtained from standard solutions of nucleotides. The linearity was checked up to 2 m*M* for each compound. Least-squares regression lines intercepted near zero with correlation coefficients greater than 0.99.

To check the methodological variability, the coefficients of variation (C.V.) were estimated from repeated determinations of a standard mixture. The intra-assay C.V. varied from 3% (ATP) to 5.5% (GMP) ($n = 10$). The detection limit ranged from 4 pmol (GMP) to 30 pmol (dATP), at a signal-to-noise ratio of 2, for 20 μl injected.

The analytical recovery of the nucleotides of interest, as determined by analysing nucleotides added to a synthetic serum containing 70 g/l protein, which was then deproteinized and washed by water-saturated diethyl ether, was between 94% (AMP) and 96% (ATP) ($n = 5$ for each compound).

Identification of compounds by LC–MS

In order to identify the structure of nucleotides from octopus retina homogenates by LC–MS, we had to change the mobile phase to make it sufficiently volatile to be compatible with thermospray interface for LC–MS. We modified essentially the buffer composition. The adopted chromatographic conditions afforded a convenient resolution of the compounds.

HPLC–MS conditions

We developed a gradient system for the elution of nucleotides. Mobile phase A was 0.1 *M* ammonium acetate, adjusted to pH 3.2 with trifluoroacetic acid. Mobile phase B was a mixture of one volume of acetonitrile with one volume of the following: methanol containing 0.05 *M* ammonium acetate adjusted to pH 3.2

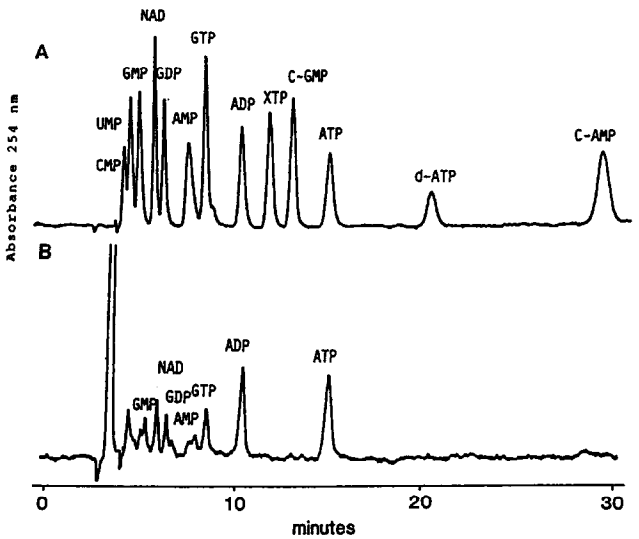

Fig. 1. Ion-pair reversed-phase HPLC separation on a 5-μm (250 mm × 4.6 mm I.D.) LiChrosorb RP-18 column of (A) a standard mixture of nucleotides containing 160 pmol of each component per injection, and (B) a homogenate of octopus retina containing 12 pmol of GMP, 38 pmol of NAD, 32 pmol of GDP, 10 pmol of AMP, 40 pmol of GTP, 140 pmol of ADP and 190 pmol of ATP.

with trifluoroacetic acid. The gradient was from 10 to 100% of B in 15 min, at a flow-rate of 1.0 ml/min.

The MS conditions were: stem temperature, 100°C; tip temperature, 200°C; vaporizer operating temperature, *ca.* 260°C; source temperature, 280°C; filament electron energy, 950 eV.

TABLE I

RETENTION TIMES AND CAPACITY FACTORS (k') OF THE SEPARATED NUCLEOTIDES

Compound	Retention time (min)	k'
CMP	4.4	0.40
UMP	4.7	0.47
GMP	5.3	0.66
NAD	6.1	0.91
GDP	6.6	1.06
AMP	7.8	1.44
GTP	8.6	1.69
ADP	10.5	2.28
XTP	12.1	2.78
C-GMP	13.4	3.19
ATP	15.5	3.84
d-ATP	20.8	5.50
C-AMP	29.6	8.25

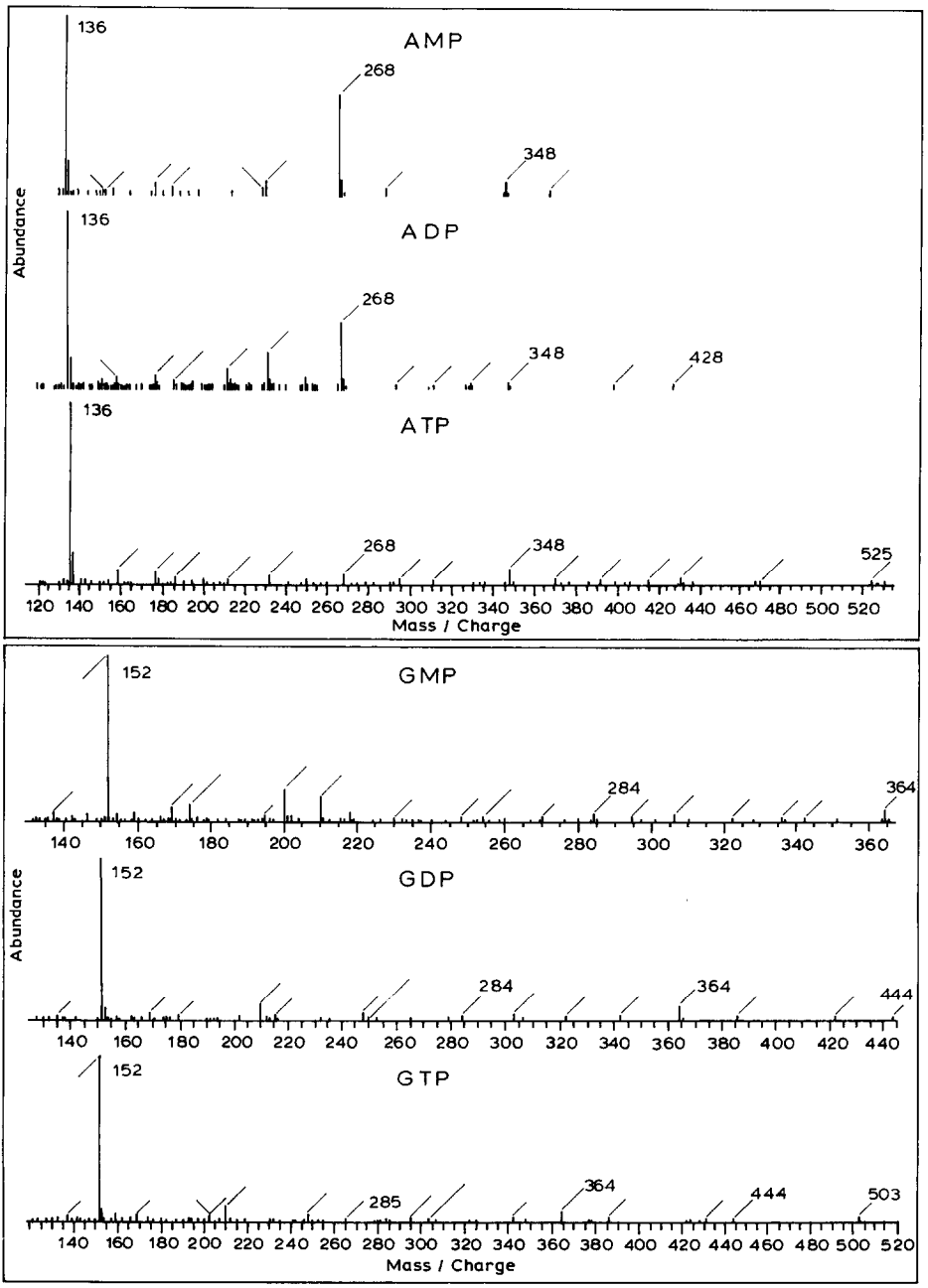

Fig. 2. Mass spectra of a standard mixture of adenylate and guanylate nucleotides.

RESULTS AND DISCUSSION

Fig. 1A shows a standard mixture containing 160 pmol of each compound separated by the method described above. Fig. 1B shows a chromatogram of

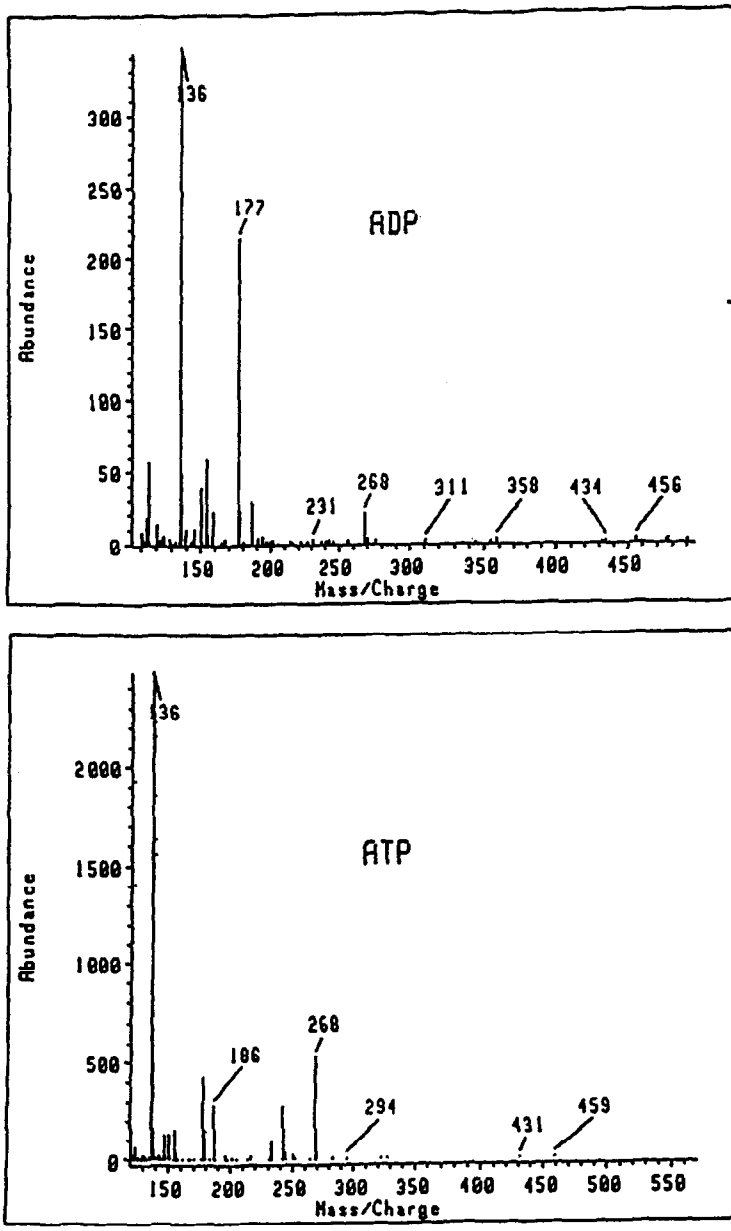

Fig. 3. Mass spectra of ADP and ATP obtained from a homogenate of octopus retina.

nucleotides in the octopus retina. Typical concentrations found were 0.05 m*M* for AMP, 0.35 m*M* for ADP, 0.60 m*M* for ATP, 0.06 m*M* for GMP, 0.2 m*M* for GDP and 0.5 m*M* for GTP. Table I gives the retention times and the capacity factors of the separated nucleotides.

A series of mass spectra (Fig. 2), obtained with the LC–MS system for the identification of nucleotides from octopus retina, shared several characteristics. For adenylate nucleotides a major fragment was measured at *m/e* 136, corresponding the base adenine. A less important fragment was measured at *m/e* 268, corresponding to the nucleoside adenosine. Other fragments of weak intensities were measured at *m/e* 348 for AMP, *m/e* 428 for ADP and *m/e* 508 for ATP. For guanylate nucleotides a major fragment was measured at *m/e* 152, corresponding to the base guanine. A less important fragment was measured at *m/e* 284, corresponding to the nucleoside guanosine. Other fragments of weak intensities were measured at *m/e* 364 for GMP, *m/e* 444 for GDP and *m/e* 524 for GTP.

The mass spectral data obtained for these compounds from homogenates of octopus retina were in agreement with those from the standard mixture. Fig. 3 shows two examples of such spectra, those of ADP and ATP.

In conclusion, an efficient separation of thirteen nucleotides can be achieved by this HPLC method. The technique is precise and reproducible for the determination of these substances in biological samples, as confirmed by MS.

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