Journal of Chromatography, 318 (1985) 433–439
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,244

Note

High-performance liquid chromatography of nucleotides and nucleotide sugars extracted from wheat embryo and vegetable seed

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(Received September 17th, 1984)

High-performance liquid chromatography (HPLC) is now routinely used in the clinical and biochemical analysis of nucleotides in urine, blood and other animal tissues[1]. However, its potential in the study of nucleotide and energy metabolism in plant tissues has yet to be exploited. The advantages of HPLC are that a number of different compounds contained within the same sample can be analysed simultaneously and with the advanced technology now available relatively crude samples can be analysed in a short space of time by automated procedures. One simple and reliable HPLC technique for analysing nucleotides from deproteinised animal tissue extracts within 20 min has been reported previously by Perret[2]. Trichloroacetic acid (TCA) extracts of wheat embryo and other plant seeds separated by this method show very complex chromatograms compared to similar extracts prepared from animal tissue[2], and contain a large number of unidentified compounds some of which are likely to be nucleotide sugars. Much of this unidentified material, in particular the compounds eluting near to the void volume, is unresolved using published HPLC separation methods and interferes with the resolution of the nucleoside monophosphates and diphosphates in the seed extracts, making quantitative analysis difficult and unreliable. We have, therefore, found it necessary to devise a method to overcome this difficulty and in the present paper we describe modifications to a separation method based on salt gradient anion-exchange chromatography which can be applied to the quantitative analysis of extracts derived from cereal, vegetable and other seeds.

MATERIALS AND METHODS

The HPLC gradient apparatus consisted of an M45 pump, a 6000A pump and a Model 660 solvent programmer (Waters Assoc., Hartford, Northwich, U.K.) and a Rheodyne Model 7125 syringe loading sample injector (Phase Separations, Queensferry, U.K.). The nucleotides eluted from the column were monitored at 254 nm using a Waters Model 440 absorbance detector connected to a Spectra-Physics 4270 computing integrator (Spectra-Physics, St. Albans, U.K.). Partisil 5 μm SAX was supplied by Whatman, Maidstone, U.K. ARISTAR grade potassium dihydrogen orthophosphate and Analar grade potassium chloride (BDH, Liverpool, U.K.) were used in the liquid phase, the water used was freshly redistilled and deionised. Ace-

0021-9673/85/$03.30

tonitrile (HPLC S grade) was supplied by Rathburn Chemicals, Walkerburn, U.K.

Extraction of wheat embryo and vegetable seeds for nucleotide analysis

Wheat embryos (50) were homogenised using a glass PTFE motor driven homogeniser in 0.4 ml of 8% (w/v) TCA 20% (v/v) methanol at 0°C, and the insoluble material removed by centrifugation for 1 min at 9000 *g* in a microcentrifuge. The soluble fraction was neutralised by extracting six times using seven volumes of water-saturated diethyl ether to remove the TCA. All operations were performed rapidly at 0°C to minimise acid hydrolysis of nucleotides and the final ether-neutralized soluble extract was stored at −70°C until required for analysis.

Vegetable seeds (50) were homogenised using a glass PTFE motor driven homogeniser in a minimal volume (0.5–1 ml) of 8% (w/v) TCA 20% (v/v) methanol at 0°C. Nucleotide extraction was then performed as described for wheat embryo tissue.

Analysis of extracts by HPLC

Aliquots (20–50 μl) of wheat embryo extract were injected onto a 20 × 0.46 cm I.D. column packed with 5 μm Partisil SAX and eluted, except where indicated in figure legends, with a concave gradient (programme 7, Waters 660 programmer) from 99% buffer A (2 m*M* potassium dihydrogen orthophosphate pH 2.75, 20% acetonitrile), 1% buffer B to 100% buffer B (1.1 *M* potassium chloride, 0.6 *M* potassium dihydrogen orthophosphate pH 3.25) at a flow-rate of 1.6 ml/min over 20 min. Conditions for separating nucleotides from vegetable seed extracts were as described for wheat embryo extracts. The eluent from the column was monitored at 254 nm and the absorbance peaks identified by co-elution using standards. Peaks were also examined by spectral analysis using a Beckman 9000 continuous flow scanner (Beckman, High Wycombe, U.K.) to check peak purity. The peak area was electronically integrated so that the amount of nucleotide present could be calculated.

RESULTS AND DISCUSSION

Fig. 1a shows the separation of an ether-neutralized TCA extract of wheat embryos separated by the original method developed by Perret[2] using 5 μm APS-Hypersil column packing material. It is evident that there is a great deal of unidentified UV absorbing material present in the wheat embryo extract most of which is eluted near to the void volume. This material is not found in extracts of animal tissues (Fig. 1d) and hence in samples of animal extraction[1] these problems of separation and quantification of nucleotides eluting in the early part of the chromatogram do not occur. However, in the analysis of plant extracts serious problems arise in that a large quantity of weakly bound unidentified UV absorbing material elutes in the same region as AMP and other nucleoside monophosphates thereby interfering with their separation and accurate estimation (Fig. 1a). Use of an alternative column packing material, 5 μm Partisil SAX, eluted with a linear salt gradient did produce a clearer separation of several nucleotides from the large peak of weakly bound UV absorbing material (Fig. 1b) although resolution of ATP and UTP was lost. The use of a concave salt gradient (Fig. 1c) gave better, although far from ideal, resolution of AMP from the large UV peak of weakly bound absorbing material and just allowed the resolution of UTP from ATP under the elution conditions employed. It

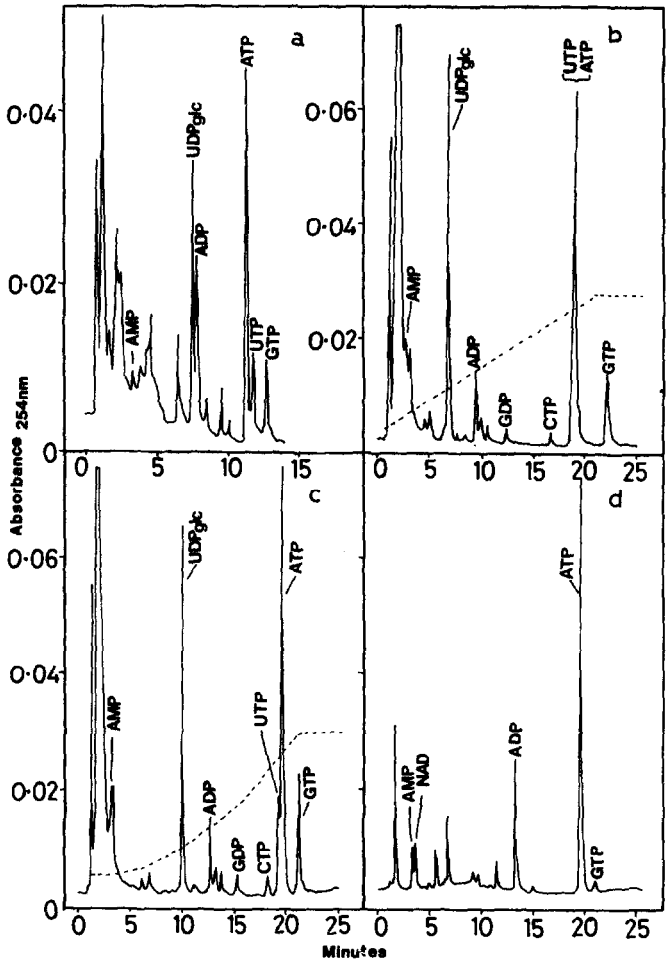

Fig. 1. Separation of extracts from plant and animal tissues on APS Hypersil and Partisil SAX columns. A neutralised extract of 5 h imbibed wheat embryos was separated using (a) the original method developed by Perret[2] on a 10 × 0.46 cm I.D. column of 5 μm APS Hypersil using a linear salt gradient from 0.04 *M* potassium dihydrogen orthophosphate pH 2.9 to 0.5 *M* potassium dihydrogen orthophosphate plus 0.8 *M* potassium chloride pH 2.9 in 13 min to elute the nucleotides, (b) a 20 × 0.46 cm I.D. column of 5 μm Partisil SAX using a linear salt gradient (– – –) from 99% buffer A (without acetonitrile), 1% buffer B to 30% buffer A (without acetonitrile), 70% buffer B (see Materials and Methods) in 20 min to elute the nucleotides, (c) conditions as for (b) except that a concave gradient (– – –) from 99% buffer A (without acetonitrile), 1% buffer B to 100% buffer B in 20 min was used to elute the nucleotides, (d) a neutralised extract from human blood was separated on a 20 × 0.46 cm I.D. column of 5 μm Partisil SAX using a concave gradient [shape as in (c)] from 99% buffer A, 1% buffer B to 100% buffer B in 20 min to elute the nucleotides.

was decided to proceed with the use of the 5 μm Partisil SAX packing material and attempt to produce elution conditions which would allow separation and quantification of all the nucleotides of interest in plant seed extracts.

AMP, a nucleoside monophosphate of particular interest, binds to the column through ionic interactions, but it is possible that much of the material interfering

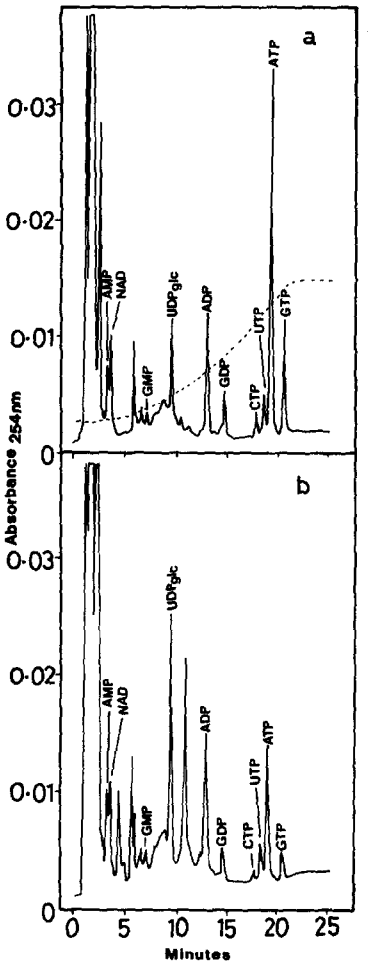

Fig. 2. Separation of extracts from wheat and lettuce seeds on Partisil SAX columns in the presence of acetonitrile in the low salt buffer. A neutralised extract of (a) 20 min imbibed wheat embryos and (b) 17 h imbibed lettuce seeds was separated on a 20 × 0.46 cm I.D. column of 5 μm Partisil SAX using a concave salt gradient [see in (a)] from 99% buffer A, 1% buffer B to 100% buffer B (see Materials and Methods). 50 μl of neutralised extract was injected on to the column in each case.

with the separation and accurate quantitative estimation of AMP is bound to the column through hydrophobic interactions. The system we have developed contains modifications which have been introduced to reduce the binding of non-polar molecules to the column and results in AMP being clearly separated from the unidentified UV absorbing material present in the plant extracts. The major modification to the original method has been to reduce reversed-phase interactions with uncapped silanol groups on the column matrix by including 20% acetonitrile in the low salt buffer A. This reduces the polarity of the mobile phase, and effectively abolishes much of the reversed-phase properties of the anion-exchange material[3].

The effect of eliminating the reversed-phase interaction is to reduce the re-

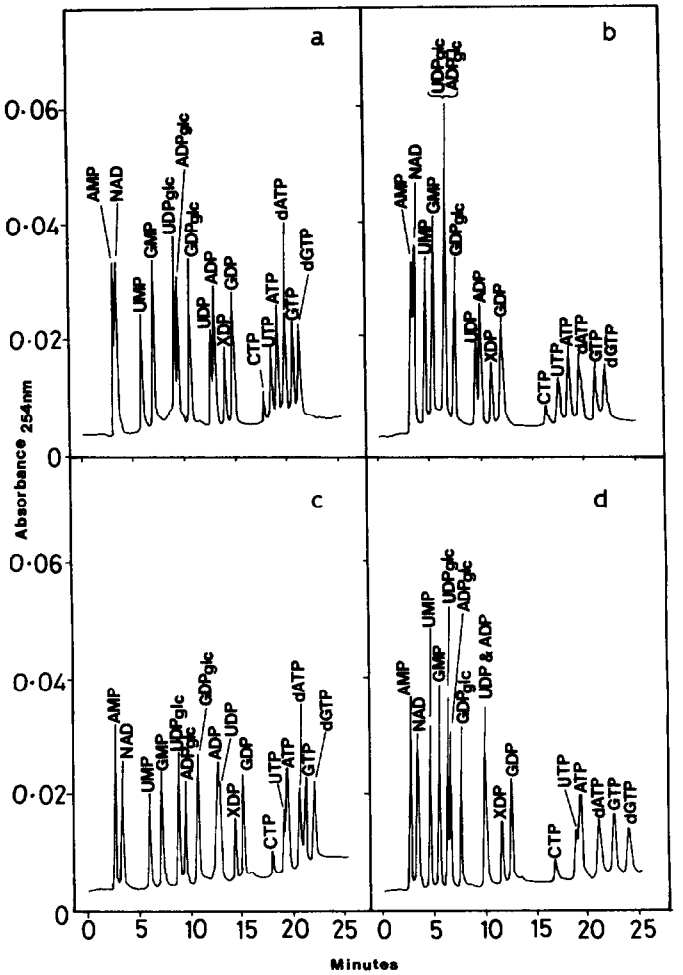

Fig. 3. Separation of a mixture of nucleotide standards on Partisil SAX columns in the presence of absence of acetonitrile in the low salt buffer. Elution of 17 nucleotides and related compounds on a 20 × 0.46 cm I.D. column of 5 μm Partisil SAX was performed using (a) a concave gradient (see Fig. 2a) from 99% buffer A, 1% buffer B to 100% buffer B in 20 min (b) a linear gradient (see Fig. 1b) from 99% buffer A, 1% buffer B to 30% buffer A, 70% buffer B in 20 min (c) a concave gradient (shape as in Fig. 2a) from 99% buffer A (without acetonitrile) 1% buffer B to 100% buffer B in 20 min (d) a linear gradient (see Fig. 1b) from 99% buffer A (without acetonitrile) 1% buffer B to 30% buffer A (without acetonitrile) 70% buffer B in 20 min.

tention of the large amount of interfering material as shown in Fig. 2a, however, it also affects the retention of anionically charged material as well. This is seen in Fig. 3 which illustrates the separation of a mixture of standard nucleotides and nucleotide sugars on a 20 × 0.46 cm I.D. column of 5 μm Partisil SAX in the presence and absence of 20% acetonitrile in buffer A. Examples of the separation of the nucleotides using either a linear or concave salt gradient are shown. It was found that the separation of uridine and adenosine di- and triphosphates was enhanced in the

presence of acetonitrile in the mobile phase and that the retention time of AMP on the column was increased by 30% under these conditions (Fig. 3). This latter observation is advantageous in the analysis of plant seed extracts since it permits the separation of the AMP peak from the considerable amount of less polar unidentified UV absorbing material contained in these extracts (Fig. 2). Efficient separation of many nucleotides and nucleotide sugars commonly found in animal and plant extracts is achieved within 22 min with a total time between injections of 30 min using a 20 × 0.46 cm I.D. column eluted with the concave gradient at a flow-rate of 1.6 ml/min. It was also demonstrated that the deoxynucleotide derivatives of ATP and GTP were clearly separated from the other nucleotides on the 5 μm Partisil SAX column under the conditions employed (Fig. 3).

The use of a concave gradient rather than a linear gradient allowed a better separation of AMP from any NAD present in the extracts when acetonitrile was present in buffer A and also permitted the separation of UDP glucose from ADP glucose, which the linear gradient failed to do. The pH conditions employed in both buffer A and buffer B are critical in the separation of both the standard nucleotide mixture and biological samples. Alteration of the pH of either buffer causes alterations in the retention times on the column of adenine and uracil nucleotides in particular and since ADP and UDP elute very close to each other, as do UTP and ATP, slight alterations in pH especially in buffer B can cause these peaks to merge together or produce poor separations. It is important that the pH of buffer B is maintained above pH 3.2 (preferably at pH 3.25) since below this pH the UTP peak begins to merge with the peak of ATP and becomes difficult to quantify but at pH 3.25 optimal separation of the two nucleotides is obtained. Additionally, in order that a consistent separation of NAD from AMP is obtained in the presence of acetonitrile, it is essential that the pH of the initial buffer A does not exceed pH 2.75 as above this pH the NAD and AMP peaks tend to merge into each other.

The method described can also be used to separate nucleotide materials in plant extracts other than wheat embryos. Fig. 2b shows the elution profile of nucleotides extracted from germinated seeds of lettuce. In addition to the cereal and vegetable seeds illustrated we have also used this method to analyse nucleotides in extracts of carrot, onion, leek, parsnip, cabbage, sugar beet, sage and rosemary seeds. In all cases high levels of weakly bound UV absorbing materials eluted from the column at the start of the run yet the AMP and NAD peaks were eluted from the column quite distinctly from this material using the elution conditions described and could be identified and accurately quantified. Previous investigations[5,6] have demonstrated that certain nucleotide and nucleotide sugar levels or their ratios in seeds during germination could be significant indicators of seed quality in terms of viability and vigour ratings. The ability to measure differences in the ratio of certain nucleotides, particularly adenine nucleotides, in the same sample during the same analysis, rather than absolute levels of individual nucleotides and to relate this ratio to seed viability and vigour ratings would eliminate any effects of seed size since seed size could be directly related to individual nucleotide levels irrespective of viability and vigour ratings of the seed. The general applicability of this HPLC analytical technique to plant tissue extracts has made it feasible to perform similar studies of nucleotide metabolism not only in cereals but also in other seeds. Exploitation of HPLC in the analysis of seed quality may open up the field of seed testing to biochemical analysis,

and augment or replace many of the present biological germination tests which are less than ideal in meeting todays demands for the rapid and accurate testing and certification of seeds.

CONCLUSIONS

The procedure described provides a rapid method for the isolation, separation and quantification of nucleotides in extracts of wheat embryo and other plant seeds. In particular, the method allows nucleoside monophosphates and NAD to be separated from the weakly bound UV absorbing material present in extracts of plant seeds which elutes in the very early part of the chromatogram interfering with the separation and quantification of several nucleotides using existing HPLC procedures. The use of HPLC facilitates a more comprehensive analysis of nucleotide metabolism than is currently possible using enzymic assays which invariably determine only one or two components, thus through the use of HPLC it becomes possible to study the overall changes and inter-relationships of nucleotide levels in seeds during germination.

ACKNOWLEDGEMENTS

The financial assistance of the Rank Prize Funds (S.A.S.) and an SERC CASE studentship (P.V.) is gratefully acknowledged. We also thank David Perret, Medical Unit, Dunn Laboratory, St. Bartholomews Hospital, London, U.K., for many helpful discussions.

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