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**Note**

# High-performance liquid chromatography of cytokinin ribonucleoside 5′-monophosphates

IAN M. SCOTT* and ROGER HORGAN

*Department of Botany and Microbiology, University College of Wales, Aberystwyth SY23 3DA (Great Britain)*

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The cytokinins, a group of naturally occurring compounds which regulate a variety of aspects of plant development, are $N^6$-substituted derivatives of adenine, which may occur as the free-base, ribonucleoside or ribonucleotide forms[1]. Although cytokinin nucleotides are common metabolites of exogenous cytokinins in plant tissues[2], and may be of significance in cytokinin biosynthesis[3], there have been few conclusive identifications of endogenous cytokinin nucleotides in contrast to numerous reports of the occurrence of base or nucleoside forms[1].

This situation is probably due to the lack of analytical methods for cytokinin nucleotides. Cytokinin purification procedures commonly involve the use of cation-exchange chromatography to separate the basic and acidic/neutral fractions of plant extracts. However, the latter fraction, in which nucleotides occur, is invariably of a viscous and pigmented nature, with a high content of compounds which are inhibitory in the bioassays widely used to screen for cytokinin activity. A common procedure is to degrade the nucleotides in the acidic/neutral fraction using enzymatic or chemical methods and then to analyse them as nucleosides or bases. However, we have found that in plant extracts these methods are unreliable. Furthermore, if employed at this stage, without any further characterization of the putative cytokinin nucleotides, this approach provides only weak evidence for an identification. For example, the poor performance of the ion-exchange step could result in a misidentification. Similarly, the cytokinin activity remaining in the aqueous fraction after butanol partitioning of an extract is often regarded as being due to nucleotides, which may then be degraded and re-analysed as nucleosides without any controls being performed. However, the partition coefficients of cytokinin glucosides are so low that there is often a residue of these compounds in the aqueous phase[4], and identifications of glucosylated cytokinin nucleotides obtained by such procedures[5] are questionable.

The identification of cytokinin nucleotides would be greatly enhanced by the use of high-resolution chromatographic procedures. High-performance liquid chromatography (HPLC) has been used increasingly in recent years for the analysis of cytokinin bases and nucleosides[6]. We have investigated the HPLC properties of some cytokinin ribonucleoside 5′-monophosphates which exhibit the full range of polarity of $N^6$-substituents found in nature. The complex nature of acidic/neutral fractions of plant extracts makes the isolation of cytokinin nucleotides a formidable task, and so

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we have attempted to develop a range of HPLC systems based on widely different separation mechanisms in order to maximize the purification achieved by a sequence of HPLC steps. Since we hope to use HPLC for preparative as well as analytical purposes, a further objective was to use solvents containing only volatile components.

## MATERIALS AND METHODS

### *Synthesis of cytokinin ribonucleoside 5′-monophosphates*

6-Chloropurine riboside 5′-monophosphate (Calbiochem) was refluxed for 3 h in butan-1-ol in the presence of a molar excess of 4-hydroxy-3-methylbut-*trans*-2-enylamine[7], 4-O-β-D-glucosyl-3-methylbut-*trans*-2-enylamine[8], or 3-methylbut-2-enylamine[9] for synthesis of the ribonucleoside 5′-monophosphates of zeatin (ZMP), zeatin-O-β-D-glucoside (ZGMP) and $\Delta^2$-isopentenyladenine (iPMP) respectively. The reaction mixture was reduced to dryness *in vacuo* and the cytokinin nucleotide product purified by column chromatography on Sephadex LH-20 (Table I), followed by paper chromatography (Table II). Product identity was confirmed by ultraviolet and mass spectrometry, HPLC and thin-layer chromatography following degradation to the riboside form using alkaline phosphatase. Yields were usually greater than 70%.

TABLE I

SEPHADEX LH-20 CHROMATOGRAPHY OF CYTOKININ NUCLEOTIDES

A column of Sephadex LH-20 (71 × 2.5 cm) was eluted with 10 m*M* formic acid at a flow-rate of 30 $cm^3$ $h^{-1}$ in the descending direction.

| *Compound* | *Elution volume ($cm^3$)* |
|---|---|
| ZGMP | 184 |
| ZMP | 222 |
| iPMP | 243 |

TABLE II

PAPER CHROMATOGRAPHY OF CYTOKININ NUCLEOTIDES

Descending chromatography on Whatman 3MM paper was carried out using butan-1-ol–acetic acid–water (12:3:5).

| *Compound* | *$R_F$ value* |
|---|---|
| ZGMP | 0.16 |
| ZMP | 0.34 |
| iPMP | 0.50 |

### *Chromatographic equipment and materials*

HPLC columns (150 × 4.5 mm I.D.) were slurry-packed at 6000 p.s.i. using a pneumatic amplifier pump. The slurry medium was acetone (for Hypersil ODS) or methanol (for Hypersil APS). Column materials were obtained from Shandon Southern, Runcorn, Great Britain.

Chromatography was carried out on a Pye LC3X system with the absorbance detector operating at 265 nm. Samples (70 $\mu$l) were introduced via an Altex 905-42 syringe-loading sample injector fitted with a 100-$\mu$l loop.

All solvents were glass-distilled prior to use. Triethylammonium bicarbonate was prepared by saturating a 2.5 *M* solution of triethylamine with carbon dioxide. Tetrabutylammonium hydroxide was obtained from Sigma.

Columns were washed and stored in methanol. Ion-pair reagent was flushed out of ODS columns with 0.1 *M* acetic acid prior to washing with methanol[10].

## RESULTS AND DISCUSSION

The ribonucleoside 5′-monophosphates of the naturally occurring cytokinins zeatin (ZMP), zeatin-O-glucoside (ZMGP), and $\Delta^2$-isopentenyladenine (iPMP) were synthesized and their HPLC properties were investigated. It was found that these compounds behaved in a much less polar fashion than the common nucleotides on reversed-phase HPLC on a $C_{18}$ bonded stationary phase, and could be resolved without the use of phosphate-buffered solvents. The resolution was marginally improved by the use of 0.1 *M* acetic acid rather than water at pH 7, presumably due to ion suppression effects. Fig. 1 illustrates the separation of the cytokinin nucleotides on a column of Hypersil ODS eluted with a gradient of increasing concentration of methanol in 0.1 *M* acetic acid. iPMP elutes much later than the zeatin-related compounds, and requires a second gradient segment for elution in a reasonable time. It is surprising that ZMP elutes before ZGMP in this system in view of the polar nature of the glucosyl moiety. Possibly there is some shielding of the charged phosphate group by the large $N^6$-substituent in the case of ZGMP.

Ion-pair reversed-phase HPLC has been used for the separation of the common nucleotides[10]. The inclusion of the volatile ion-pair reagent tetrabutylammonium hydroxide in the reversed-phase system greatly altered the chromatographic

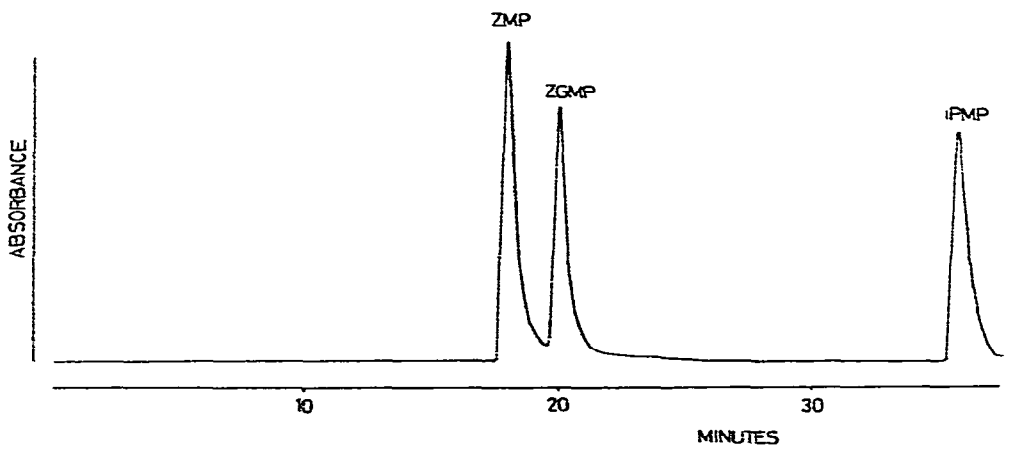


Fig. 1. Separation of cytokinin nucleotides by reversed-phase HPLC. A column (150 × 4.5 mm I.D.) of Hypersil ODS was eluted at a flow-rate of 2 $cm^3$ $min^{-1}$ with a 2-segment linear gradient of methanol in 0.1 *M* acetic acid. Segment 1 = 0–15% methanol over 30 min, segment 2 = 15–80% methanol over 10 min. ZMP, ZGMP and iPMP are the ribonucleoside 5′-monophosphates of zeatin, zeatin-O-$\beta$-D-glucoside and $\Delta^2$-isopentenyladenine respectively.

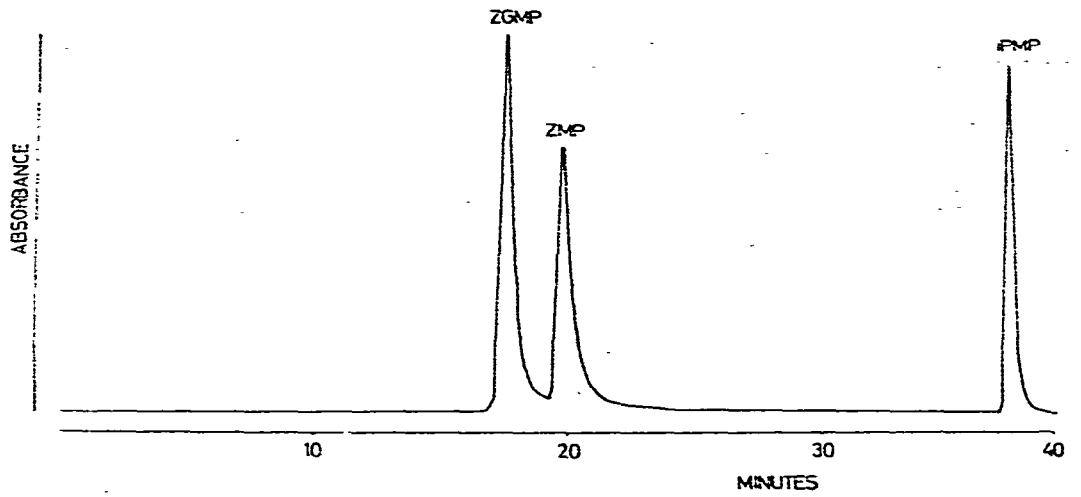


Fig. 2. Separation of cytokinin nucleotides by ion-pair reversed-phase HPLC. A column (150 × 4.5 mm I.D.) of Hypersil ODS was eluted at a flow-rate of 2 $cm^3$ $min^{-1}$ with a 2-segment linear gradient of methanol in 0.1 *M* acetic acid containing 2.5 m*M* tetrabutylammonium hydroxide. Segment 1 = 15–30% methanol over 30 min, segment 2 = 30–90% methanol over 10 min. Abbreviations as in Fig. 1.

properties of the cytokinin nucleotides, making available a contrasting separation system. As shown in Fig. 2, a much higher concentration of methanol is required to elute the cytokinin nucleotides in the ion-pair system, and the elution sequence of ZMP and ZGMP is reversed. Presumably the counterion nullifies the charge on the phosphate group so that the polar nature of the glucosyl moiety of ZGMP becomes of greater significance in this system.

HPLC of nucleotides in a strong anion-exchange system would involve the use of involatile buffer salts[11], but it was found that the cytokinin nucleotides could be

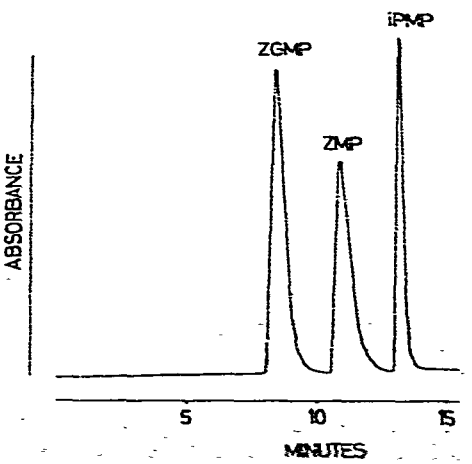


Fig. 3. Separation of cytokinin nucleotides by ion-exchange HPLC. A column (150 × 4.5 mm I.D.) of Hypersil APS was eluted at a flow-rate of 2 $cm^3$ $min^{-1}$ with a linear gradient of 100 m*M* triethylammonium bicarbonate (0–40% over 20 min) in 10 m*M* ammonium bicarbonate. Abbreviations as in Fig. 1.

separated on a column of Hypersil APS, an aminopropyl-bonded silica with weak anion-exchange properties, using volatile buffers. Fig. 3 shows the separation achieved using a gradient of increasing proportion of 100 m*M* triethylammonium bicarbonate in 10 m*M* ammonium bicarbonate. Complete though slightly reduced resolution was also achieved using 1 m*M* acetic acid in place of ammonium bicarbonate. This system probably operates by gradual suppression of the ionization of the amino groups of the stationary phase as the pH of the solvent rises. The salts in the effluent may be removed by a few evaporations with methanol.

The cytokinin nucleoside di- and triphosphates seem to occur in some tissues[2] but not others[3]. The analysis of these compounds by HPLC would probably require the use of involatile buffer salts.

These HPLC systems have been used to identify chromatographically ZMP as an endogenous cytokinin (detected by bioassay) and as a metabolite of [$^{14}$C]zeatin in *Vinca rosea* crown gall tissue. We therefore anticipate that HPLC will provide an efficient and reliable method for the analysis of cytokinin nucleotides and should help to remedy the lack of knowledge of these potentially important compounds.

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