Precolumn Fluorescence Derivatization of Nucleosides for Mono- and Oligonucleotides

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The determination of mono- and oligonucleotides has been required not only for their characterization¹, but also for evaluating their physiological roles.^{2,3}

We previously reported that 2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran (OMB-COCl) reacted in a benzene-pyridine mixture with all kinds of nucleosides at the 5'-hydroxyl group to produce the corresponding fluorescent esters, which could be separated by reversed-phase high-performance liquid chromatography (HPLC).⁴ The purpose of this study was to establish an HPLC method for the quantification of mono- and oligonucleotides by modifying the above-mentioned techniques. The conditions of the derivatization reaction and HPLC separation were investigated using adenosine-3'-monophosphate (Ap), uridine-3'-monophosphate (Up), 2'-deoxyadenosine-3'-monophosphate (dAp) and thymidine-3'-monophosphate (Tp) as model mononucleotides, 2'-deoxyadenylyl(3'-5')thymidine [d(AT)] as a dinucleotide and 2'-deoxyadenylyl(3'-5')-2'-deoxyguanylyl(3'-5')-2'-deoxycytidylyl(3'-5')-thymidine [d(AGCT)] as a tetranucleotide.

Experimental

Chemicals and HPLC system

OMB-COCl was obtained from Dojindo Laboratories (Kumamoto, Japan). Ap, cytidine-3'-monophosphate (Cp), guanosine-3'-monophosphate (Gp), Up, dAp, 2'-deoxycytidine-3'-monophosphate (dCp), 2'-deoxyguanosine-3'-monophosphate (dGp), Tp, and d(AT) were purchased from Sigma (St. Louis, MO, USA) and d(AGCT) from Takara Shuzo (Kyoto, Japan).

The HPLC system and its operation conditions were the same as described previously⁴, except that the mobile phase was a mixture of acetonitrile and a 50 mM McIlvaine buffer (a solution containing 50 mM citric acid and 50 mM sodium dihydrogen phosphate, pH 3.0) (3:7, v/v).

Derivatization procedure

An aqueous mixture of nucleotides (0.1 – 1.0 ml) placed in a screw-capped vial (3.5 ml) was lyophilized. To the lyophilizate was added a mixture of pyridine and 3 mM OMB-COCl in benzene (1:3, v/v) (0.5 ml). The vial was tightly closed and heated at 100°C for 90 min in the dark. After cooling, the reaction mixture was dried in a stream of nitrogen at 37°C; the residue was dissolved in the mobile phase for HPLC. The resulting solution (20 µl) was subjected to HPLC.

Results and Discussion

Higher temperatures allowed the derivatization reaction to proceed more rapidly in the 60 – 120°C range. At 100°C the peak heights reached maxima upon heating for 60 – 120 min; heating at 100°C for 90 min was employed in the procedure.

Pyridine, an accelerator of the derivatization reaction, provided maximum peak heights for the nucleotides at concentrations of 2.5 – 4.1 M in the reaction mixture; 3.1 M was used in the procedure. The most intense peaks for the nucleotides (concentrations, 8.75 nmol ml⁻¹ each in the reaction mixture) were obtained at OMB-COCl concentrations of 2 – 10 mM; 3 mM was used. OMB-COCl afforded singlet peaks for the respective nucleotides under the recommended derivatization conditions; MOB-COCl possibly reacted with the nucleotides at the 5'-hydroxyl group.

The fluorescent products from the six nucleotides could be satisfactorily separated by reversed-phase HPLC on a TSKgel ODS-80T_M (TOSOH, Tokyo, Japan) with isocratic elution using a mixture of acetonitrile and a 50 mM McIlvaine buffer (pH 3.0) (3:7, v/v). Figure 1 shows a typical chromatogram obtained with the fluorescent products from the six nucleotides under the recommended HPLC conditions (for the retention times see Table 1).

The relationships between the peak heights and the

Fig. 1 Chromatogram of the fluorescent products from nucleotides. The peaks for nucleotides are shaded. Peaks: 1=Up, 2=Ap, 3=Tp, 4=dAP, 5=d(AT), 6=d(AGCT), others=the reagent blank. Concentrations (on column): 35 pmol each.

amounts of nucleotides (10 species, listed in Table 1) were linear up to 1 nmol per 20- μ l injection volume, respectively. The precision was established by repeated determinations ($n=10$) using a mixture of the nucleotides (concentrations, 8.75 nmol ml⁻¹ each). The relative standard deviations (%) were 1.4(Ap), 0.6(Cp), 1.5(Gp), 2.0(Up), 1.3(dAp), 1.5(dCp), 1.8(dGp), 3.0(Tp), 4.8[d(AT)] and 3.0[d(AGCT)]. The detection limits were 0.8–6 pmol per 20- μ l injection volume (Table 1).

Table 1 Retention times and detection limits ($S/N=3$) of the fluorescent products from nucleotides

Nucleotide	Retention time/min	Detection limit (pmol on column)
Ap	2.8	4.0
Cp	2.6	4.5
Gp	2.6	5.0
Up	2.4	3.0
dAp	3.8	3.8
dCp	3.8	4.7
dGp	3.5	6.0
Tp	3.0	3.0
d(AT)	11.8	1.2
d(AGCT)	22.3	0.8

The method should be usable for the determination of those nucleotides derived from polynucleotides by a nuclease-mediated reaction.

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