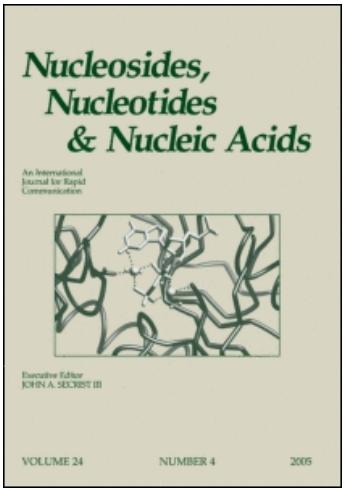

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Determination of Nucleosides and their Adducts with RP-HPLC and (Laser-)Fluorescence Detection

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DETERMINATION OF NUCLEOSIDES AND THEIR ADDUCTS WITH RP-HPLC AND (LASER-)FLUORESCENCE DETECTION

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INTRODUCTION

Interactions of carcinogens or their metabolites with the DNA of the target organ is generally supposed to be an essential step in chemical carcinogenesis. With most carcinogens, however, more than one interaction product is formed and quantitative and qualitative analyses of the different modes of interaction form a necessary tool in order to decide whether one or more of these interactions are involved in the tumor formation. Furthermore factors which may be critical in the genesis of cancer by such compounds include the specific positions which are modified in DNA bases, the relative proportions of the lesions and the persistence of these adducts in DNA bases following carcinogen exposure [1]. The determination of femtomole quantities of carcinogen-DNA adducts is thus quite important in studies designed to examine mechanisms operative in carcinogenesis induced by carcinogenic agents.

Radioactive chemical carcinogens have provided most of our present knowledge about interactions between carcinogens and components of biological systems. Their use had made possible the detection of covalent binding of chemicals to biological macromolecules in vitro and in vivo. The requirement of radioactive carcinogens restricts carcinogen-DNA binding studies to chemicals that are readily available in isotopically labeled form, i.e. a minute fraction of all potentially mutagenic or carcinogenic chemicals. To extend the scope of carcinogen-DNA binding studies, Randerath et al. [2-6] developed an alternative method for the investigation of the binding of chemicals to DNA, which does not require radioactive test chemicals. In this approach, radioactivity (32P) is being incorporated into DNA constituents after exposure of the DNA to a non-radioactive binding chemical. The presence of chemically altered nucleosides is shown by the appearance of extra spots on t.l.c.'s of digests of the chemically modified DNA, as detected by autoradiography.

So, while the sensitivity of this 32P-postlabelling method is mainly depended on the amount of time that the t.l.c. plates are monitored by autoradiography, the required separation of the diverse modified nucleosides is restricted.

The development of high-performance liquid chromatography (HPLC) has recently facilitated the isolation and quantification of the nucleic acid constituents in biological fluids and tissues; separations which previously required several hours by open-column methods can be achieved rapidly using HPLC. With on-line detection systems, the

characterization as well as the accurate quantification of the solutes of interest can be accomplished [7]. But in the case of on-line detection of 32P-postlabelling method developed by Randerath is, because of the relatively short time to monitor, not sensitive enough.
Therefore a new method is developed which combines the technique of in vitro labelling of nucleosides with a fluorescent reagent with the methods of reversed phase high-performance liquid chromatography (RP-HPLC) and (laser-)fluorescence detection.
While most of the commercially available reagents react with primary and/or secondary amino-groups, these reagents are not applicable for the carcinogenic DNA binding studies mentioned here. In general the nucleosides are altered via their bases; so the hydroxy-groups on the sugar part of the nucleosides are the only available functional groups which remain for the derivatization by a fluorescent reagent.
The labelling method presented here is based on the derivatization of nucleosides with 1-anthroylnitril in the presence of quincuclidine. This method is introduced by Goto et al. [8-13] for the determination of steroids, like 6β-hydroxycortisol and serum bile acids.
This paper mainly deals with the use of 1-anthroylnitril for the derivatization of nucleosides, followed by separation with RP-HPLC and quantification with (laser-)fluorescence detection.

EXPERIMENTAL

High Performance Liquid Chromatography

The LC system was composed of two Millipore-Waters (Etten-Leur, the Netherlands) model 6000A solvent delivery modules, which were controlled by a Millipore-Waters (Etten-Leur, the Netherlands) Automated Gradient Controller and a Millipore-Waters (Etten-Leur, the Netherlands) injector, model WISP 710B. The column effluent was monitored with a UV-detector, model Spectroflow 757 (Applied Bio Systems, Maarssen, the Netherlands) and a Schoeffel (Applied Bio Systems, Maarsen, the Netherlands) model FS 970 LC Fluorometer. The data were recorded with the Beckman, PeakPro software system.
For experiments with laserfluorescence detection, a Spectra Physics (Eindhoven, the Netherlands) model 2025 Argon ion laser was used.
A LC-PAH (5 μm) column (4.6 x 250 mm) (Supelco, Leusden, the Netherlands) and 6 ml disposable WP PEI (NH) columns (10 SPE™ System of J.T. Baker, Phillipsburg) were used at ambient temperature.

Materials

The following materials were used:
Milli-Q-water (Millipore-Waters, Etten-Leur, the Netherlands), methanol (HPLC grade, Rathburn), acetonitril (HPLC-grade, Aldrich), dimethylsulphoxide (p.a. grade, Fluka AG), 1-anthroyl nitril (synthesized according to Goto et al. [8] by ITC, TNO, Zeist, the Netherlands), quinuclidine.HCl (97%, Janssen Pharmaceutica), 2'deoxyguanosine, 2'deoxycytidine, 6-methyladenosine, 1-methylguanosine, 3-methylthymidine, thymidine and 2'deoxyadenosine (Sigma).

Procedures

A. Derivatization of 2'deoxyadenosine with 1-anthroylnitril in acetonitril

20 μg of 2'deoxyadenosine is derivatized by adding 200 μl of a 3.5 mM 1-anthroylnitril solution in acetonitril and 100 μl of a 0.07 M quinuclidine solution in acetonitril. The reaction vial is then placed in a water bath at 60°C for 120 minutes.

The obtained reaction mixture is directly analysed with RP-HPLC and UV- and fluorescence detection.
After removing the excess of reagent by adding 100 µl methanol to the reaction mixture, followed by placing the vial for 5 minutes in the above mentioned water bath, the reaction mixture is again analysed with RP-HPLC.
In order to determine whether a quantitative analysis is possible after derivatization with 1-anthroylnitril, 0.1, 0.5, 1, 2, 2.5, 4 and 5 µg of 2'deoxyadenosine are derivatized according to the procedure described above.
In this case the excess of reagent is not removed by adding an amount of methanol to the reaction mixture.
Analyses are performed with RP-HPLC and fluorescence and laser-fluorescence detection.

B. Derivatization of nucleosides and methylated nucleosides with 1-anthroylnitril in acetonitril

A mixture of 9.9 µg 2'deoxyadenosine, 15.1 µg 2'deoxycytidine, 12.5 µg 2'deoxyadenosine, 9.7 µg 6-methyladenosine, 9.2 µg 1-methylguanosine and 6.3 µg 3-methylthymidine is derivatized with 1-anthroylnitril according to the method described above, without adding methanol to remove the excess of reagent.
Analyses are performed with RP-HPLC and fluorescence detection.

C. Derivatization of thymidine with 1-anthroylnitril in dimethylsulphoxide (DMSO)

16 µg of thymidine is derivatized by adding 200 µl of a 0.02 M 1-anthroylnitril solution in DMSO and 20 µl of a 0.07 M quinuclidine solution in DMSO. The vial is placed in a water bath at 90°C for 60 minutes. The excess of reagent is removed by adding 100 µl methanol to the vial, followed by placing the vial in a water bath for 5 minutes.
The reaction mixture is analysed with RP-HPLC and fluorescence detection immediately before and after removal of the excess of reagent.

D. Removal of the excess of reagent with solid phase extraction (SPE)

9.8 µg of 2'deoxyadenosine is derivatized in duplo by adding 200 µl of a 0.02 M 1-anthroylnitril solution in DMSO and 20 µl of a 0.07 M quinuclidine solution in DMSO to each vial. The vials are placed in a water bath at 90°C for 60 minutes. The obtained reaction mixtures are mixed, and one part is used for analysis with RP-HPLC. The other part is used for the removal of excess of reagent with SPE.
In order to remove the excess of reagent an anionexchange (a polyethyleneimine, PEI, bonded silica gel) cartridge column is used.
After washing the PEI column with two column volumes (= 2 x 5 ml) DMSO, the part separated for the SPE-treatment of the reaction mixture is brought on the PEI column. The reaction products of 2'deoxyadenosine are eluted with 1 ml DMSO, and are directly analysed with RP-HPLC.

RESULTS AND DISCUSSION

When the derivatization of nucleosides with 1-anthroylnitril is carried out in acetonitril, optimal results are obtained under the reaction conditions described in procedure A. About 90% of the nucleosides are derivatized under these conditions. With NMR- and IR-spectroscopy, it is shown that mainly the 5' position of the sugar part of the nucleosides reacts with 1-anthroylnitril and forms the corresponding ester.
The chromatograms for the analysis of 2'deoxyadenosine after the derivatization with 1-anthroylnitril through RP-HPLC and UV- and fluorescence detection are demonstrated in Figure 1. The chromatographic conditions are given in the subscription of Figure 1. As

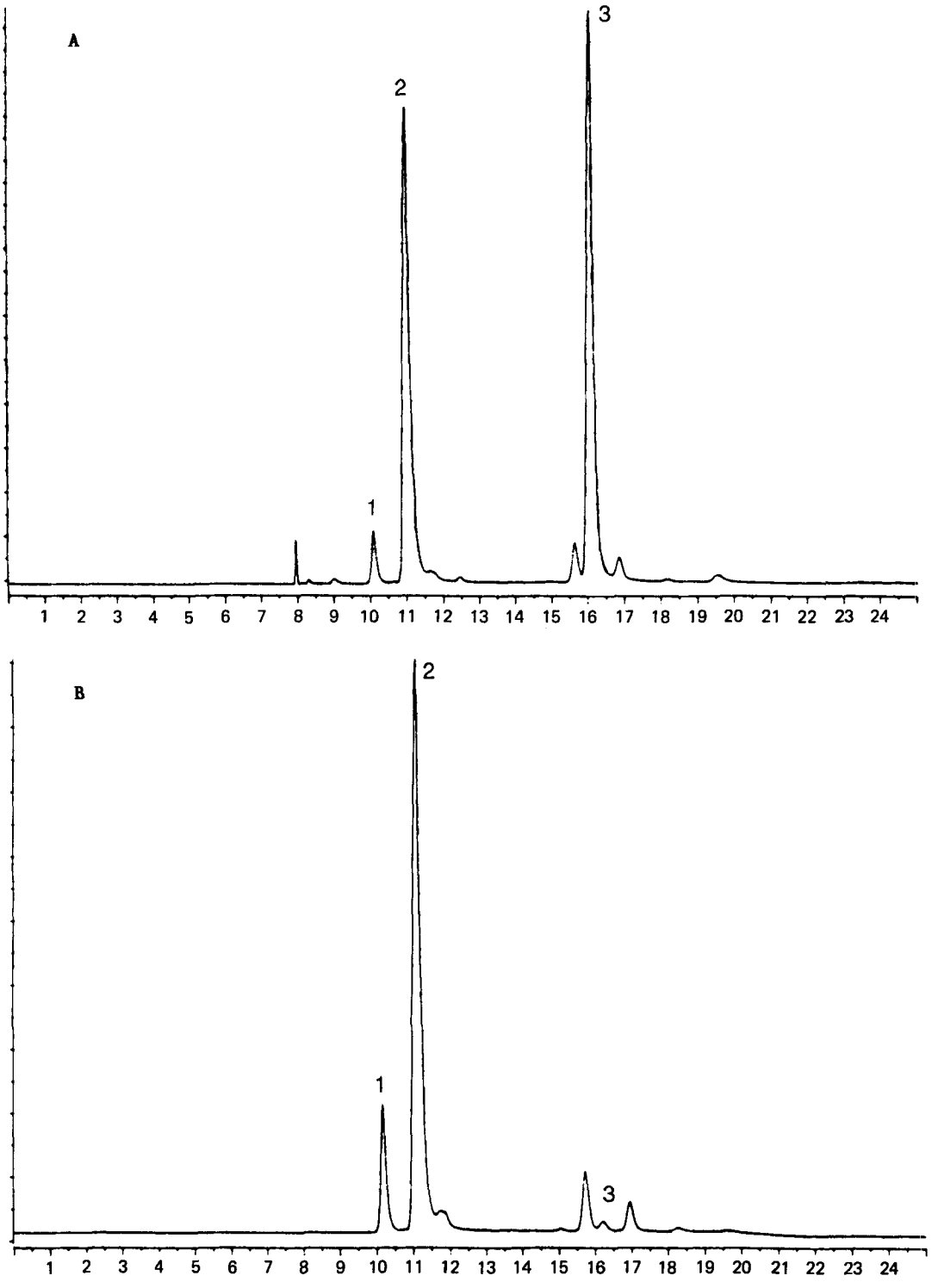

Figure 1 *Derivatization of 2'deoxyadenosine with 1-anthroylnitril before removing the excess of reagent. Mobile phase: from water to acetonitril in 20 minutes following curve 4, 1 ml/min, injection volume: 5 µl.*
A = UV-detection (254 nm), B = fluorescence detection (λ_{ex} = 370 nm, λ_{em} > 470 nm).
1 = derivate of 2'deoxyadenosine, 2 = carboxylic acid of 1-anthroylnitril, 3 = 1 anthroylnitril.

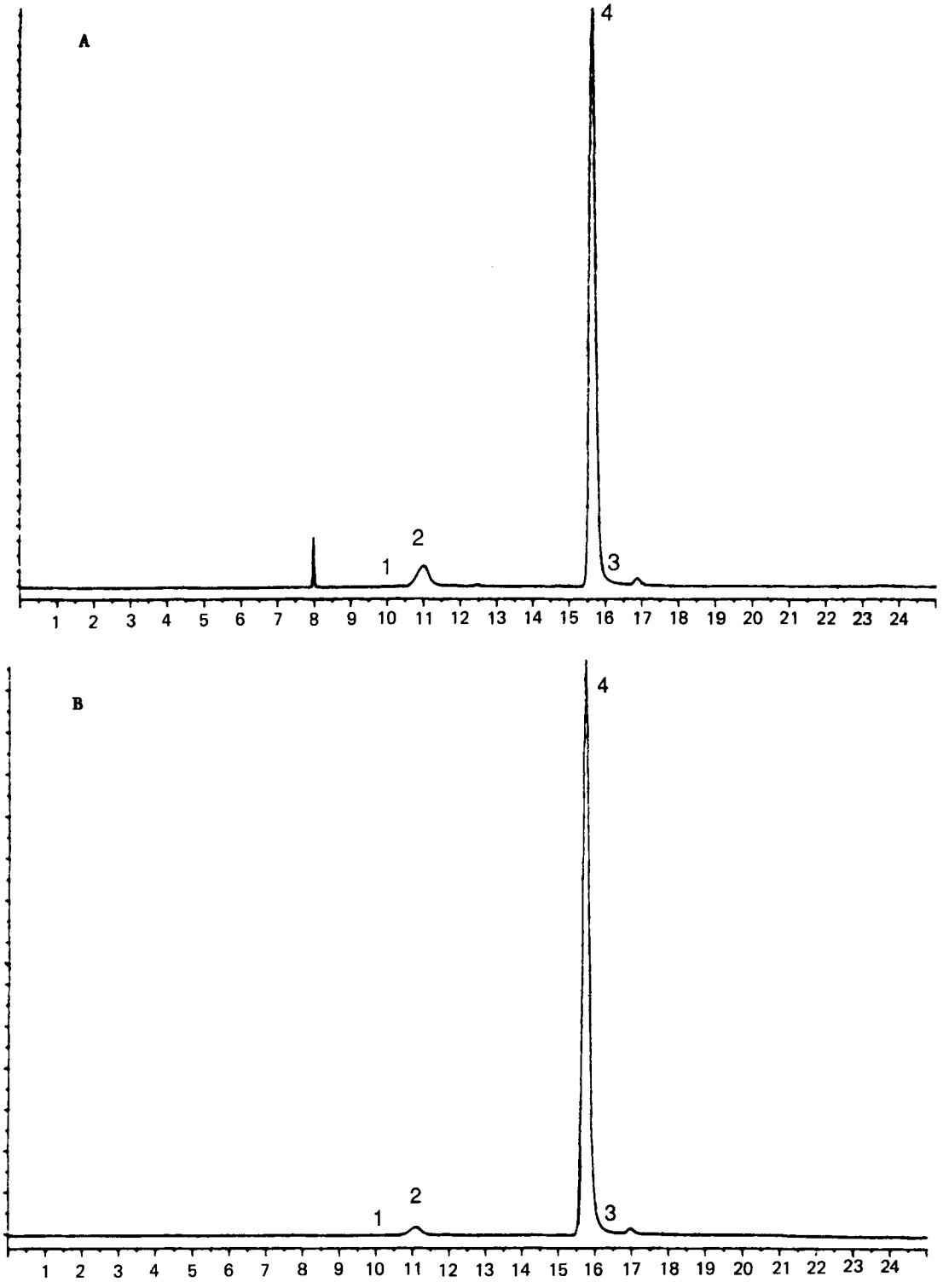

Figure 2 *Derivatization of 2'deoxyadenosine with 1-anthroylnitril after removing the excess of reagent(for the chromatographic conditions see Figure 1).*
1 = derivate of 2'deoxyadenosine, 2 = carboxylic acid of 1-anthroylnitril, 3 = 1-anthroylnitril, 4 = methylester of 2'deoxyadenosine.

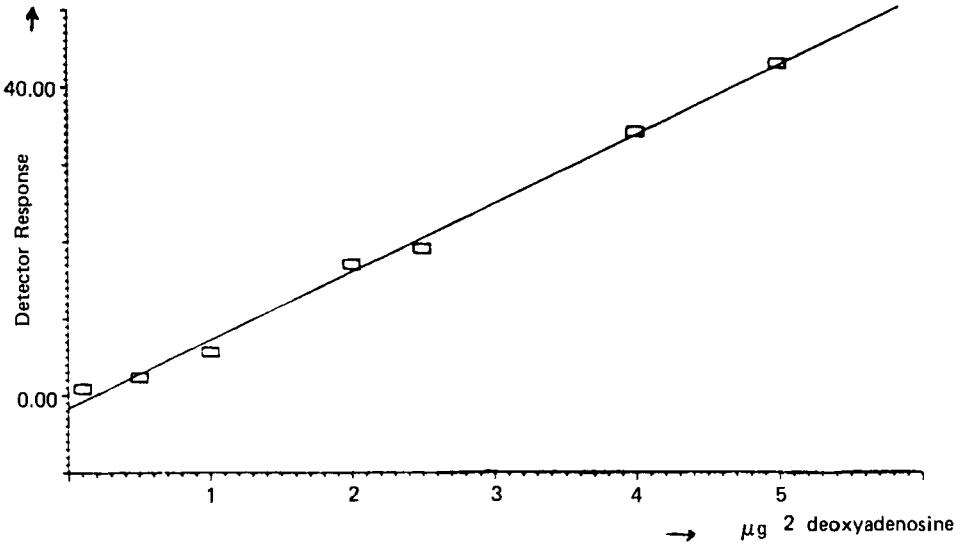

Figure 3 *Calibration curve for the determination of 2'deoxyadenosine after derivatization with 1-anthroylnitril.*

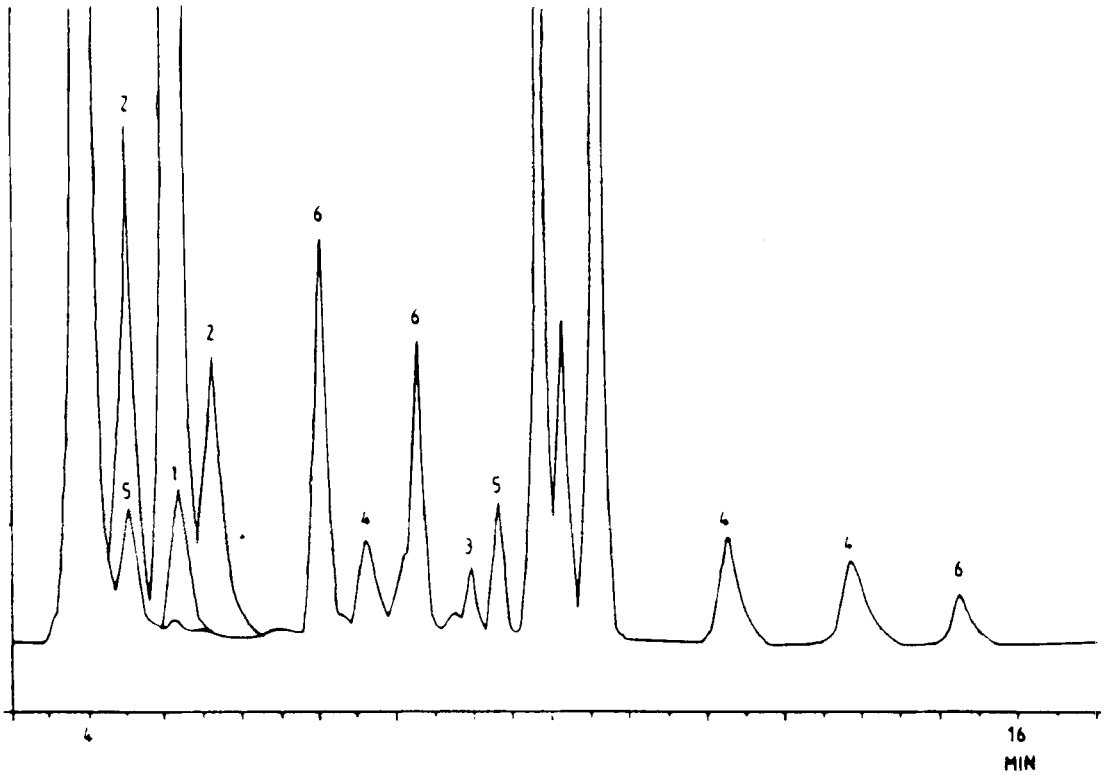

Figure 4 *Derivatization of nucleosides and methylated nucleosides with 1-anthroylnitril (for the chromatographic conditions see Figure 1).*
1 = 2'deoxyguanosine, 2 = 2'deoxycytidine, 3 = 2'deoxyadenosine, 4 = 6-methyladenosine, 5 = 1-methylguanosine and 6 = 3-methylguanosine.

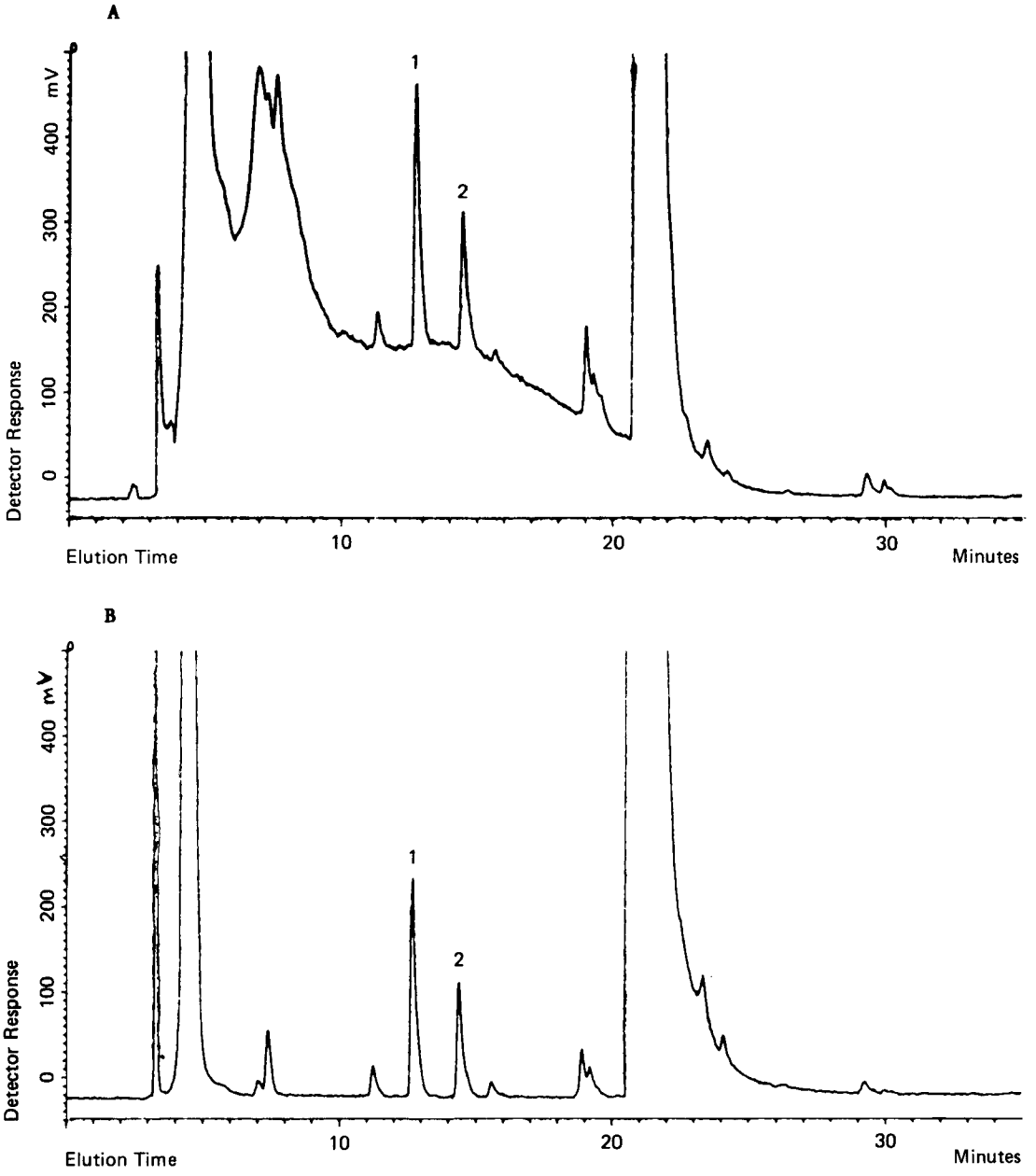

Figure 5 *Derivatization of thymidine with 1-anthroylnitril.*
A = before removing, B = after removing the excess of reagent.
Mobile phase = 30 v/v% acetonitril linear in 5 minutes to 45 v/v% acetonitril and linear in 20 minutes to acetonitril, 1 ml/min, injection volume = 20 µl, fluorescence detection (λ_{ex} = 370 nm, λ_{em} > 470 nm).
1 and 2 are reaction products of thymidine with 1 anthroylnitril.

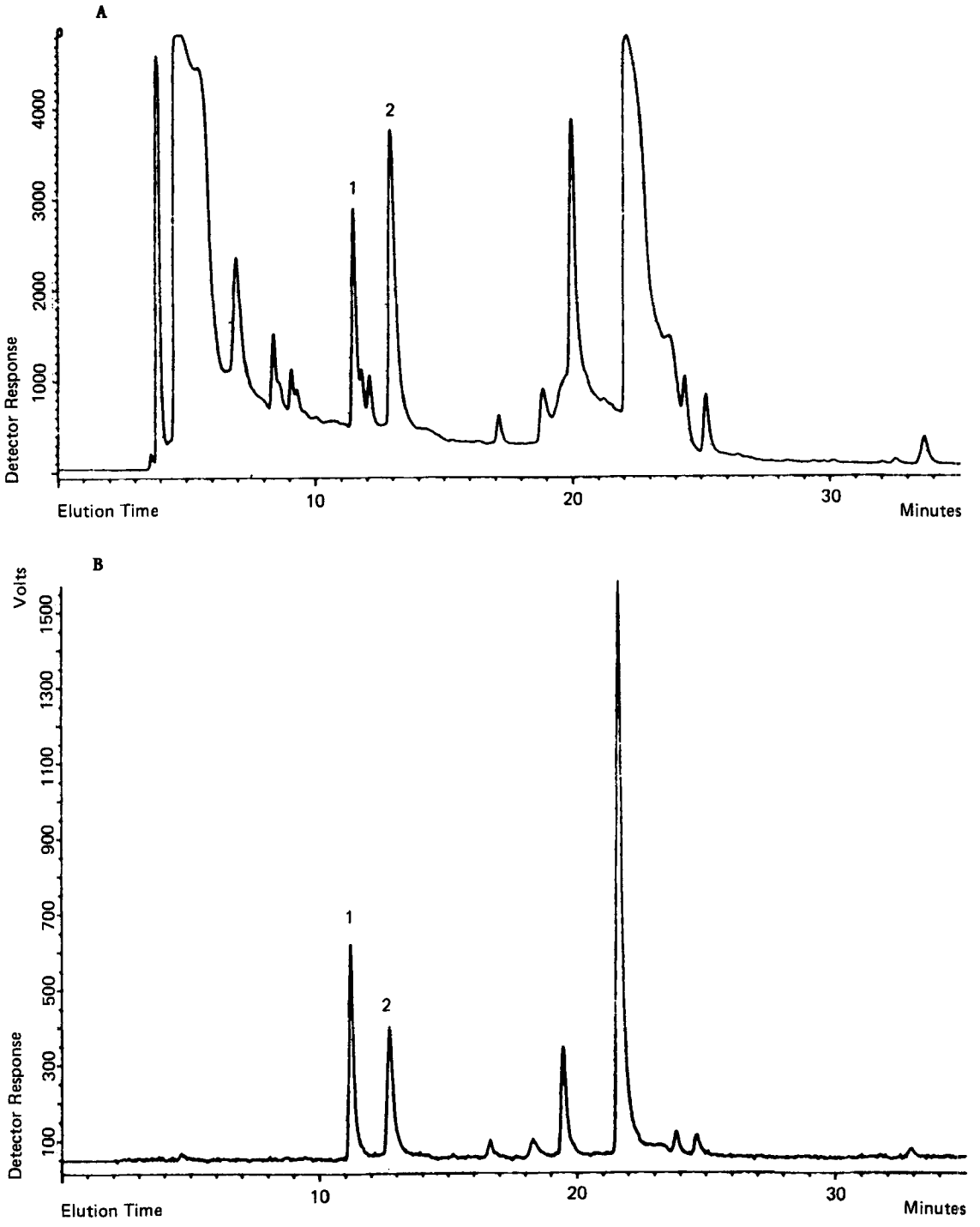

Figure 6 *Removal of excess of reagent with SPE (for the chromatographic conditions see Figure 5). A = before, B = after removing excess of reagent and C = additional elution with 1 ml DMSO after eluting the reaction products of 2'deoxyadenosine with 1 ml DMSO. 1 and 2 are reaction products of 2'deoxyadenosine with 1 anthroylnitril.*

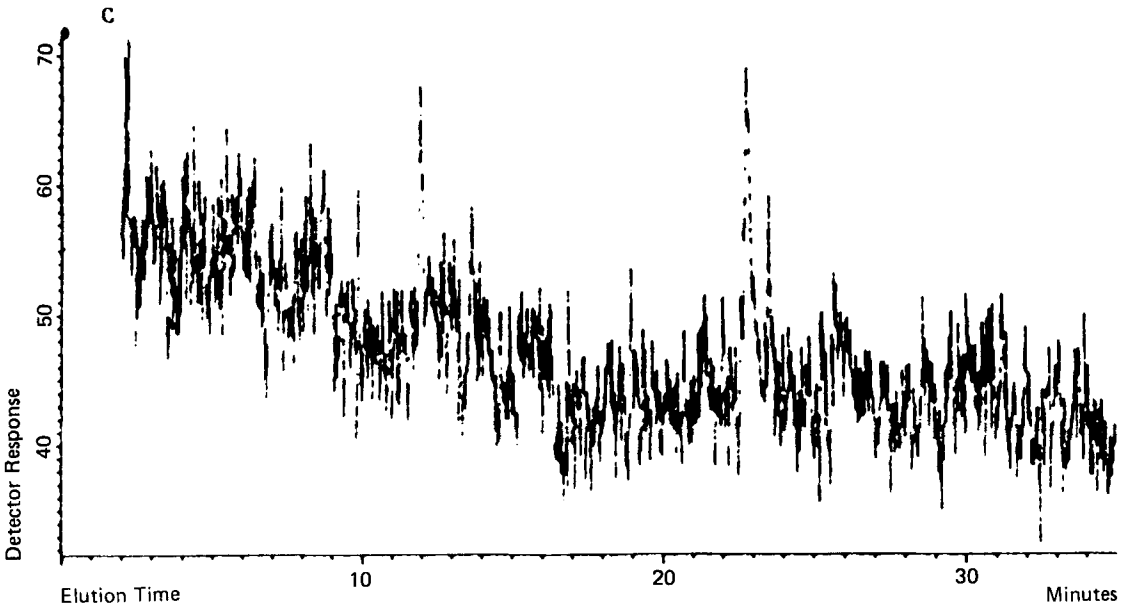

Figure 6 Continued

shown in this figure the reaction product of 2'deoxyadenosine with 1-anthroylnitril elutes after 10 minutes. While 1-anthroylnitril is very sensitive towards water, a certain amount of 1-anthroylnitril reacts with water during the derivatization and analysis with RP-HPLC and corresponding carboxylic acid is formed. This compound and 1-anthroylnitril elutes after 11 and 16 minutes respectively. Quinuclidine gives no signal when UV- or fluorescence detection is used.

Although it cannot be read from Figure 1, the presence of carboxylic acid can disturb the analysis. To avoid this Goto et al. [8-13] used methanol to remove the excess of 'reactive' 1-anthroylnitril. Corresponding methylester is formed, which can easily be separated from the reaction products of the nucleosides. As demonstrated in Figure 2, 1-anthroylnitril converts in the presence of methanol relatively fast and efficient to the corresponding methylester. But, as can be seen in Figure 2, 1-anthroylnitril as well as the reaction product of 2'deoxyadenosine, probably via a transesterification, disappears. Consequently it is not possible to remove the excess of reagent with methanol, and other solutions have to be searched for to solve this problem, one of which might be an adjustment of the chromatographic conditions. However, we have first investigated the possibility of a quantitative analysis and the detection limits after the derivatization with 1-anthroylnitril. For that reason different amounts of 2'deoxyadenosine were derivatized with 1-anthroylnitril and analysed with RP-HPLC and fluorescence- and laserfluorescence detection. As demonstrated in Figure 3, the conclusion was arrived at that 2'deoxyadenosine reacts quantitatively with 1-anthroylnitril.

The detection limit for the determination of 2'deoxyadenosine under these conditions is calculated as 1 pMol. Although detection with laserfluorescence is not yet optimised at our laboratory, detection limits of 5 fMol 2'deoxyadenosine are already achieved.

Another important condition, is that the nucleosides as well as their adducts must react with 1-anthroylnitril. Therefore different sorts of nucleosides are derivatized and analysed with

RP-HPLC and fluorescence detection. The obtained chromatogram is demonstrated in Figure 4. As shown in this figure some of the nucleosides provide more than one reaction product. Although we were not able to identify all the peaks in the chromatogram with NMR and IR, it was evident that in this case 1-anthroylnitril did not react with the amino groups of the base part of the nucleosides. So probably the 3' position and the 2' position, in case it is substituted by a hydroxyl group, of the sugar part of the nucleoside react with 1-anthroylnitril as well. Some of the reaction products are difficult to separate with RP-HPLC, although many different chromatographic conditions are tried, but, moreover, the present excess of 1-anthroylnitril still remains a serious problem (see Figure 4). Especially when detection through laserfluorescence is performed, a raised base line, caused by the formation of the carboxylic acid during the chromatographic proces, makes it still impossible to detect low quantities of nucleosides.

As discussed earlier, it is not possible to remove the excess of reagent with methanol. Although there is not yet a valid explanation for this phenomenon, yet the decision was made to investigate whether the solvent affects the stability of the formed esters. Different kinds of solvents were tried, all with polarities comparable with acetonitril and with a low quantity of water. Because of the positive results with dimethylsulphoxide (DMSO), we decided to continue with this solvent. Because a certain amount of 1-anthroylnitril always reacts with water present in the solvent, we also decided to use a 20 mM instead of a 3.5 mM 1-anthroylnitril solution, in order to obtain more reproducable results.

The chromatogram for the analysis of thymidine after the derivatization with 1-anthroylnitril and RP-HPLC and fluorescence detection is demonstrated in Figure 5A. As shown in this figure the reaction products of thymidine elute at about 12.8 and 14.3 minutes respectively. The raised base line, caused by the formation of the carboxylic acid, can clearly be seen in this figure. Yet, obviously, it disappeared when methanol was used to remove the excess of reagent (see Figure 5B). However, a certain amount of carboxylic acid always remains because of the water present in the solvent and the samples.

Figure 5B shows that the reaction products of thymidine are significantly more stable in DMSO. Although it is not demonstrated in this paper, nucleosides and their adducts react quantitatively with 1-anthroylnitril in acetonitril as well as in DMSO. Their reaction products are at least stable for 1 week at ambient temperature.

The presence of an excess of reagent (as a methylester and/or as a carboxylic acid) is still unacceptable, although significant improvements are made. So, other methods had to be tried to remove the excess of reagent. Application of the solid phase extraction (SPE) for this problem seemed to be a valid choice.

After several experiments, application of a polyethyleneimine (PEI) bonded silica gel cartridge column appeared to give the best results.

The Figures 6A and B clearly demonstrate the removal of the excess of reagent with the PEI column. The difference in ratio between peak 1 and 2 in both figures, is due to the fact that in Figure 6A, peak 2 coelutes with another, unknown, compound. Figure 6C demonstrates that the reaction products of 2'deoxyadenosine are eluted with 1 ml DMSO.

Although not presented in this paper, clean ups with SPE in duplo show remarkable reproducable results. SPE can also be easily applied for mixtures of derivatized nucleosides and their adducts.

However, this method has one major disadvantage. While dilution is inevitable, and DMSO is very difficult to concentrate, the gain in sensitivity can be lost when using this method.

CONCLUSIONS

Quantitative determination of nucleosides and their adducts with RP-HPLC and (laser-)fluorescence detection after derivatization with 1-anthroylnitril in the presence of quinuclidine gave satisfactory results. Detection limits of 5 fMol nucleoside are already achieved. Mainly the presence of anthracene-1-carboxylic acid, derived from the reaction of an excess of the reagent with water, disturb the analysis. When the reaction was carried

out in DMSO the removal of the excess of the reagent with methanol was possible and a significant improvement was made. Whereas the application of SPE to remove the excess of reagent showed remarkable good results, although a dilution of the sample has to be taken into consideration.
Finally, it is recommended to separate and fractionate the nucleosides before derivatization with 1-anthroylnitril, because it is very difficult to separate the various reaction products from the different nucleosides.

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