

Improved ^{32}P -postlabelling assay for the quantification of the major platinum–DNA adducts

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For the improvement of chemotherapy with platinum (Pt)-containing drugs a sensitive assay to detect the induced Pt-DNA adducts is needed. Therefore, the ^{32}P -postlabelling assay, described by Blommaert and Saris (*Nucleic Acids Res.*, 1995, 23, 1300–1306), to detect the major adducts Pt-GG and Pt-AG has substantially been improved and compared with ELISA and AAS. For the quantification of the adducts, TpT was added as an internal standard immediately after isolation of the Pt-adducts from digested DNA samples. It was found that ^{32}P -labelling of both GpG and ApG, the dinucleotides obtained after deplatination of the adducts, was equally efficient as that of TpT. To isolate the Pt-adducts on basis of a positive charge, the pH of DNA digests was adjusted to ~3 prior to separation by strong cation-exchange chromatography. For the subsequent deplatination a volume of only 12 μl of 0.2 M NaCN was used, which did not interfere with the following labelling step. The quantification of the ^{32}P -labelled dinucleotides was performed by phosphorimaging of spots after separation on TLC as well as by ^{32}P -counting of fractions collected after separation by HPLC. The method was used to determine adduct levels in *in vitro* cisplatin-treated DNA and in DNA isolated from cisplatin-treated cultured cells, tumor xenografts from cisplatin-treated mice, and from white blood cells and (tumor) tissues from cisplatin-treated patients. The results show a significant correlation with the adduct levels as determined with atomic absorption spectroscopy (high levels) or with specific antibodies (low levels). This assay appears to be useful for the determination of low levels of Pt-adducts in small DNA samples as present in clinical specimens such as blood and

***Abbreviations:** AAS, atomic absorption spectroscopy; cisplatin, *cis*-diamminedichloroplatinum(II); ApG, 2'-deoxyadenylyl (3'→5')-2'-deoxyguanosine; CpC, 2'-deoxycytidylyl (3'→5')-2'-deoxycytidine; GpG, 2'-deoxyguanylyl (3'→5')-2'-deoxyguanosine; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; G-Pt-G, *cis*-Pt(NH₃)₂(dG)₂; HPLC, high performance liquid chromatography; N, nucleosides; NaAc, sodium acetate; NaCN, sodium cyanide; P_i, inorganic ^{32}P -phosphate; PNK, T4-polynucleotide kinase; Pt, Platinum; Pt-AG, *cis*-Pt(NH₃)₂d(ApG); Pt-GG, *cis*-Pt(NH₃)₂d(GpG); SCX, strong cation-exchange; TLC, thin layer chromatography; TpT, thymidylyl (3'→5')thymidine.

tumor tissue, but also in buccal mucosal cells and fine needle aspirates.

Introduction

Cis-diamminedichloroplatinum(II) (cisplatin*) is widely used for chemotherapy of a broad range of solid tumors. It is generally accepted that the antitumor activity of cisplatin and other platinum (Pt) antitumor drugs is due to the binding of these drugs to cellular DNA. Cisplatin can form mono-adducts and various types of bifunctional adducts (2). The main adduct formed is *cis*-Pt(NH₃)₂d(GpG) (Pt-GG), with Pt bound to two adjacent guanines. Another major intrastrand crosslink is *cis*-Pt(NH₃)₂d(ApG) (Pt-AG), in which the Pt is bound to adenine and an adjacent guanine. The other bifunctional adducts are *cis*-Pt(NH₃)₂(dG)₂ (G-Pt-G), the intrastrand adducts in which Pt is bound to two guanines separated by one or more other nucleotide(s) and the interstrand crosslinks on two guanines in opposite strands.

There are several methods to determine the total amount of Pt-adducts in isolated DNA (3), of which atomic absorption spectroscopy (AAS) is the most common (4,5). Also various immunochemical techniques are available using polyclonal antibodies directed against platinated DNA (6–10). None of these systems, however, is able to quantify the various DNA adducts separately. For this purpose an immunochemical method has been developed by Fichtinger-Schepman and colleagues (2,11,12), using polyclonal antibodies directed against the different types of adducts as present in digested DNA samples (ELISA method). Although this method is very sensitive, drawbacks are the rather large amounts of DNA needed, the limited availability of the rabbit antisera and the fact that these antibodies are only suitable to detect adducts induced by cisplatin and carboplatin. Also, this method is rather laborious.

Therefore, there was a need for a more generally applicable and sensitive method to determine adduct levels in small samples of platinated DNA. Such an assay was recently developed by Blommaert and Saris (1), using a ^{32}P -postlabelling technique. In this assay the DNA is digested, followed by isolation of the Pt-adducts on the basis of their positive charge, their deplatination with sodium cyanide (NaCN) and finally the labelling of the resulting dinucleotides with [γ - ^{32}P]ATP. This deplatination appeared to be a crucial step to enhance the sensitivity of the assay, because the dinucleotides are labelled with a much higher efficiency than the platinated adducts themselves (13,14). Under the assay conditions published previously (1), fmol amounts of the two major intrastrand adducts Pt-GG and Pt-AG could be measured in small amounts of DNA (~10 μg), after deplatination. The G-Pt-G and mono-adducts formed upon cisplatin treatment are not detected in this assay because they are not substrates for the labelling enzyme due to the lack of a 3'-phosphate. In the assay as described by Blommaert and Saris (1), reproducible quantita-

tive analysis was impossible due to the absence of an internal standard. Furthermore, the recovery of the adducts was only 30%. In the present paper we report on the improvement of the assay by optimizing the isolation of the adducts on the strong cation-exchanger. In addition, an internal standard for the labelling reaction was added and the deplatination step was modified, so that a subsequent purification could be omitted. For the quantification of the ^{32}P -labelled products thin-layer chromatography (TLC) in combination with phosphorimaging was used, as well as high performance liquid chromatography (HPLC) with ^{32}P -counting of the collected column fractions. The improved assay was found to be quite suitable to determine adduct levels in *in vitro* cisplatin-treated DNA, in DNA isolated from cisplatin-treated cultured cells, in DNA from tumor xenografts in mice given cisplatin therapy, as well as in DNA from human white blood cells and (tumor) tissues of cisplatin-treated patients.

Materials and methods

Reference compounds

The dinucleotides GpG, ApG and TpT were purchased from Sigma (St Louis, MO) and dissolved in distilled water. The Pt-GG and Pt-AG adducts were isolated from cisplatin-treated salmon sperm DNA (2) and quantified on the basis of their Pt content by AAS. G-Pt-G was prepared by incubation of deoxyguanosine with cisplatin (2:1 ratio) for 5 h at 50°C in the dark and purified as described by Fichtinger-Schepman and coworkers (15).

Treatment of DNA with cisplatin

Salmon sperm DNA (Worthington Biochemical Corporation, Freehold, NJ), dissolved in 10 mM sodium phosphate pH 7.2 (1 mg/ml), was incubated for 5 h at 50°C with cisplatin (Platinol, Bristol Myers Squibb B.V., Woerden, The Netherlands) at concentrations ranging between 0 and 42.7 nM. Unbound and mono-functionally bound cisplatin were inactivated by overnight dialysis against 0.1 M NH_4HCO_3 at room temperature, followed by dialysis against water at 4°C. The amounts of bifunctional adducts Pt-GG and Pt-AG in these DNAs were determined by competitive ELISA in the chromatographed, digested samples, as described by Fichtinger-Schepman *et al.* (12). An additional DNA sample was incubated with 3.3 μM cisplatin to obtain sufficiently high adduct levels in order to determine these also with AAS (16). Furthermore, part of this sample and untreated DNA were postincubated with 10 mM thiourea (Aldrich Chemie, Steinheim, Germany) for 1 h at 37°C in order to see whether this procedure, introduced recently as an alternative for the NH_4HCO_3 treatment for the rapid and more reliable inactivation of unbound and mono-functionally bound cisplatin (17), could have an effect on the postlabelling results.

Treatment of cultured cells with cisplatin

The IGROV-1 human ovarian carcinoma cell line was originally described by Benard *et al.* (18). Cells were routinely cultured in RPMI 1640 medium with HEPES (pH 7.4) and phenol red, supplemented with 10% bovine calf serum, 10 mM NaHCO_3 , 2 mM glutamine, gentamycin (45 $\mu\text{g}/\text{ml}$), penicillin (110 IU/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were frequently monitored for mycoplasma contamination.

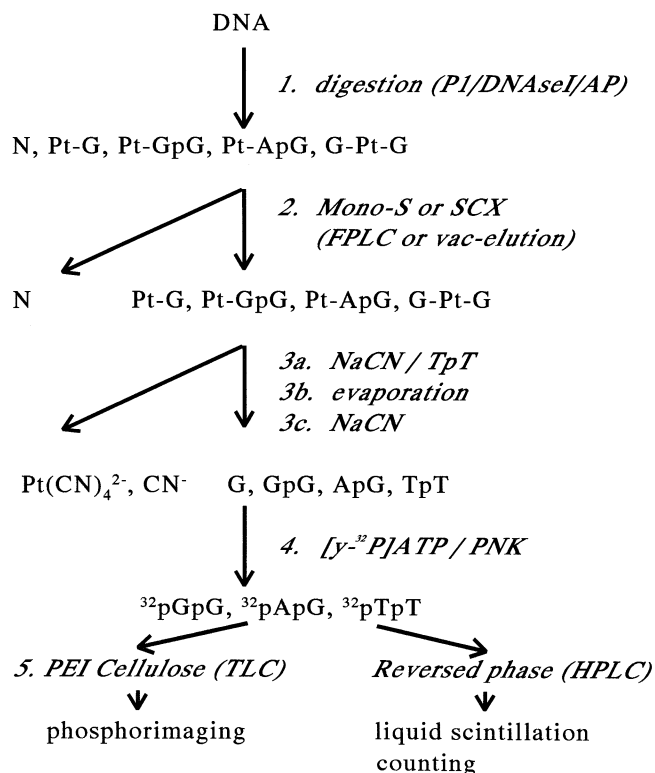
In order to study the formation of GpG and ApG intrastrand adducts in these cells, 10^6 cells were incubated with 0–66.7 μM cisplatin for 2 h at 37°C. Subsequently, medium was removed, cells were washed twice with phosphate-buffered saline (PBS) and trypsinized. After centrifugation cell pellets were stored at –20°C until DNA isolation.

Treatment of tumors with cisplatin

Human head and neck squamous cell carcinomas (HNSCC) were grown as xenografts in nude mice (19). Subsequently, these tumors were treated *ex vivo* with 1.7 mM cisplatin for 1 h. To obtain *in vivo* treated samples, nude mice bearing human ovarian carcinomas (OVCAR1) were treated intravenously with 5 mg cisplatin/kg and killed at different time periods after treatment. In addition to the tumors, liver and kidney tissues also from these mice were stored at –20°C until DNA isolation.

DNA isolation

DNA was isolated as described previously (12,17). For the cisplatin-treated IGROV-1 cells and blood samples from cisplatin-treated patients, the isolation procedure with high-salt extraction was used as described by Miller *et al.* (20). When cisplatin-containing samples were postincubated in drug-free



Scheme I. A schematic outline of the ^{32}P -postlabelling assay. The isolated DNA is digested into unmodified nucleosides (N) and Pt-adducts by the enzymes P1-nuclease (P1), DNase I and alkaline phosphatase (AP) (1). Then, the adducts are purified by strong cation-exchange (SCX) chromatography (2), and collected in tubes containing NaCN and the internal standard TpT (3a), whereafter the samples are dried in vacuo (3b). Subsequently, after addition of NaCN to a total of 12 μl 0.2 M NaCN, the adducts are deplatinated by incubation for 2 h at 65°C (3c), whereafter they are postlabelled (4), separated and quantified (5).

medium for several hours, the inactivation step with thiourea was not necessary because reactive mono-adducts were no longer present in platinated DNA after that period (17).

DNA digestion

Two methods were applied for the digestion of DNA to deoxynucleosides (see Scheme I, no. 1). First, DNA was digested as described by Blommaert and Saris (1) with the exception that after the digestion only 1/100 instead of 1/10 volume of 1 M Tris-HCl pH 9.0 was added. This volume was sufficient to reach the pH optimum of calf intestinal alkaline phosphatase (Life Technologies, Breda, The Netherlands). Under these conditions a complete digestion of the major intrastrand crosslinks to Pt-GG and Pt-AG dinucleotides will be obtained (2).

A second method to digest DNA was by dissolving 10–100 μg DNA in 50 μl of 50 mM ammonium acetate (NH_4Ac , pH 5.0) to which 6 units nuclease P1 (Boehringer Mannheim, Germany) and 1 mM ZnCl_2 (pH 8.2) were added. After an incubation at 55°C for 2 h, 10 μl of 1 M Tris-HCl (pH 8.2) with 10 mM MgCl_2 and 50 units DNase I (Boehringer) were added, after which the incubation was prolonged for 2 h at 37°C. Finally, 20 units alkaline phosphatase (Boehringer) were added, the pH was adjusted to 9.0 and the digestion mixture was incubated at 37°C for another 2 h for samples containing <25 μg , and overnight for samples with more DNA. The efficacy of this digestion procedure and the amount of DNA were determined by HPLC analysis on a C_{18} -column (Inertsil ODS-80A, 150 \times 4.6 mm, 5 μm) eluted with 0.1 M NH_4Ac (pH 5.5)/10% MeOH at a flow rate of 1.0 ml/min. In this system the nucleosides dC, dG, T and dA elute at 2.0, 3.1, 3.9, and 6.1 min, respectively. The exact amount of DNA could be calculated from the integrated dA peak (using $\epsilon_{260} = 15,400$ l/mol/cm).

Purification and concentration of cisplatin-DNA adducts

Separation of the platinated adducts from the unmodified deoxynucleosides was achieved by strong cation-exchange (SCX) chromatography, either with the strong cation-exchange column Mono-S coupled to an FPLC system (Pharmacia Biotech Benelux, Roosendaal, The Netherlands) (Scheme I, no.

2), or by use of SCX solid-phase extraction cartridges (Lichrolut SCX, 200 mg; Merck, Darmstadt, Germany) and a vacuum manifold (Vac-Elut 10, Applied Science Group, Emmen, The Netherlands). Also GpG and ApG dinucleotides from unplatinated DNA regions, if any present, will be separated from the platinated products during this step.

FPLC-method. Before the purification of the platinated DNA adducts on the Mono-S column, the pH of the DNA digest was lowered to ~3. After injection of the sample, the column was eluted for 4 min with buffer A to remove the unmodified nucleosides. Then, the adducts were eluted with buffer B for 3 min at 1 ml/min, followed by washing of the column at a flow rate of 2 ml/min for 3.5 min with buffer B and 4 min with buffer A. The amount of DNA in the sample was calculated by comparison of the total peak areas of the unmodified nucleosides with those of known samples of digested salmon sperm DNA. Synthetic G-Pt-G was used as a reference to determine the elution position of the Pt-adducts. The retention time of this reference adduct was ~6.2 min. The adducts, present in the middle 1-ml fraction of buffer B, were collected in siliconized tubes already containing 0.5 or 0.6 pmol of TpT as internal standard and 1.2 μmol NaCN (no. 3a in Scheme I). Then the samples were dried in vacuo (Scheme I, no. 3b). The presence of NaCN in the samples prevents digestion of the internal standard by nucleases that co-elute with the adducts from the column (data not shown).

SCX solid-phase extraction. On the vacuum manifold 10 samples can be processed simultaneously. Before the adduct purification, the required number of SCX cartridges were washed as follows: 2×1 ml water, 2×1 ml MeOH, and again 2×with 1 ml water. The cartridges were equilibrated by rinsing twice with 1 ml of 0.05 M Tris–HCl (pH 3.0). Prior to loading, the pH of the DNA digests was also adjusted to ~3 by addition of 0.8 volume of 0.05 M HCl. After loading the samples, unmodified deoxynucleosides were eluted with 8 1-ml washes of 10 mM NH₄-formate buffer pH 6.0 (buffer A), followed by elution of the platinated products with 2 portions of 500 μl 0.25 M NH₄OH (buffer B). The latter two eluted fractions of 500 μl were collected in one tube containing 1.2 μmol NaCN and 0.5 or 0.6 pmol of TpT as the internal standard (no. 3a in Scheme I). Samples were dried in vacuo (Scheme I, no. 3b).

Deplatinatation of cisplatin–DNA adducts

After drying, the mixture of purified Pt-adducts, reference TpT and 1.2 μmol NaCN was dissolved in 12 μl 0.1 M NaCN (final concentration 0.2 M NaCN, pH ~10) and incubated for 2 h at 65°C in order to remove Pt from the adducts (Scheme I, no. 3c). The samples were stored at –20°C until postlabelling.

³²P-Postlabelling

In 12 μl of the deplatinated-adduct solution containing 0.2 M NaCN, the pH was lowered with 4 μl of 3 M sodium acetate (NaAc; pH 5.4) to ~8.2 which is the optimum pH for the enzyme T4-polynucleotide kinase (PNK; 10 U/μl, 3'-phosphatase free; Boehringer). Because of the volatility of HCN, formed upon lowering of the pH (pK_a = 9.3), the following steps were performed in a hood to avoid inhalation. To each sample, 3 μl of the labelling-mixture was added followed by a 40-min incubation at 37°C. The labelling-mixture consisted of 0.5 μl [γ-³²P]ATP (3000 Ci/mmol, 3.3 pmol/μl; Amersham Life Science, Amersham, UK), 2 μl 10 times concentrated kinase buffer (Boehringer) and 0.5 μl PNK (no. 4 in Scheme I).

Separation and quantification of the ³²P-labelled dinucleotides

The separation of the radioactively labelled products can be performed in two ways, either by TLC or HPLC (Scheme I, no 5).

TLC. Polygram Cel 300 PEI TLC sheets (Macherey-Nagel, 20 [width]×40 [height] cm, Düren, Germany) were prewashed with 50% MeOH and air-dried. After labelling, 5 μl of the mixtures were applied on the sheets and run for ~5 h with 1.5 M NH₄-formate buffer (pH 4.0), after which the solvent front had reached ~5 cm underneath the top of the sheet. After drying of the sheets, the amount of radioactivity of each spot was quantified by use of a PhosphorImager 425 with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The dinucleotides GpG, ApG and TpT were labelled with equal efficiency (see below). Therefore, the original amounts of Pt-GG and Pt-AG adducts in the digested DNA samples could be calculated by comparing the background-corrected radioactivity of the ³²pGpG and ³²pApG spots with that of the standard TpT after labelling (i.e. ³²pTpT) according to the equation:

$$\text{amount of adduct} = \frac{(^{32}\text{P content adduct})}{(^{32}\text{P content pTpT})} * \text{amount TpT}$$

HPLC. For the separation of the ³²P-labelled products by HPLC, the samples were diluted to 150 μl with water. Depending on the amount of adducts present, a maximum of 50 μl was injected onto the C₁₈-column. Products were separated during isocratic elution with 0.1 M KH₂PO₄ (pH 4.0)/2.5% MeOH. Fractions of 0.5 ml were collected and Cerenkov radiation was counted. The total amount of each compound was calculated from its total background-corrected radioactivity compared to that of the reference compound TpT, as described for the TLC method, above.

Results

The ³²P-postlabelling assay to determine the two major Pt-DNA intrastrand crosslinks Pt-GG and Pt-AG, as introduced by Blommaert and Saris (1), is a well-designed and sensitive method. However, various steps in the protocol still needed to be improved. The results of our experiments to optimize the assay and its application on clinical relevant samples are described here.

Purification of cisplatin-DNA adducts

The Pt-adducts in the DNA digests were separated from the unmodified deoxynucleosides by strong cation-exchange chromatography, by use of either the Mono-S column or the SCX-cartridge. In our hands, the Pt-adducts did not bind to the cation-exchanger unless the pH of the DNA digest was lowered to ~3. Following removal of unmodified nucleosides 85–100% of the adducts could be recovered after elution with NH₄OH. They could be collected in 1 ml of eluent, as was verified by AAS measurements when reference Pt-GG and Pt-AG adducts were applied onto the column in a mixture with 0.5 g of digested salmon sperm DNA. The Pt-adducts were collected in tubes containing TpT as internal standard and 1.2 μmol NaCN. NaCN was added to maintain a high pH during the subsequent drying step, when NH₃ was evaporated, in order to prevent any digestion of the internal standard by traces of co-eluted nucleases (data not shown).

Deplatinatation of the cisplatin-DNA adducts

The final concentration of 0.2 M NaCN, contained in only 12 μl, was sufficient to obtain complete deplatinatation, as was concluded from the complete shifts of the UV-absorbing peaks during chromatography on a Mono-Q anion-exchange column upon deplatinatation of 0.5 nmol of the adducts Pt-GG and Pt-AG (not shown). The presence of such a small amount of NaCN, after adjustment of the pH, did not affect the PNK enzyme activity during the ³²P-labelling (see below). However, due to the presence of the cyanide, Pt-determination in the Mono-Q fractions with AAS, to double-check the deplatinatation, was not possible. From the shifts in the retention times it could be concluded that the deplatinatation of Pt-AG was faster than that of Pt-GG. A complete deplatinatation of Pt-AG, but not of Pt-GG, was already found when a concentration of 0.1 M NaCN was used.

³²P-Postlabelling

The mixture of deplatinated products and NaCN could directly be used for the postlabelling. This was concluded from the equally efficient labelling of 1 pmol of ApG and GpG standards in the presence or absence of 12 μl 0.2 M NaCN. This amount of NaCN was found not to affect the reaction when 1/3 volume of 3 M NaAc pH 5.4 was added prior to the labelling in order to lower the pH to ~8.2, which is the optimum pH for the labelling enzyme PNK (data not shown).

Separation of the ³²P-labelled dinucleotides

This has been achieved in two different ways. First, by use of chromatography on PEI cellulose TLC sheets. Even when the whole ³²P-postlabelled sample (19 μl) was spotted, a good separation of the reaction products ³²pGpG, ³²pApG, ³²pTpT from excess of [γ-³²P]ATP and inorganic ³²P-phosphate (P_i) could be obtained as shown in Figure 1. In a second method the labelled products were separated on a C₁₈-column by HPLC (Figure 2). This method resulted in elution of ³²pGpG after 6 min, ³²pApG after 9 min, and of ³²pTpT after 19 min. Excess of [γ-³²P]ATP was eluted after 2 min.

Labelling efficiency of the dinucleotides ApG and GpG

To study the kinetics of the labelling reaction, various amounts (0–500 fmol) of the dinucleotide ApG were postlabelled in the presence of 500 fmol TpT and with or without 50 fmol of GpG. After quantification with the TLC method, linear dose-

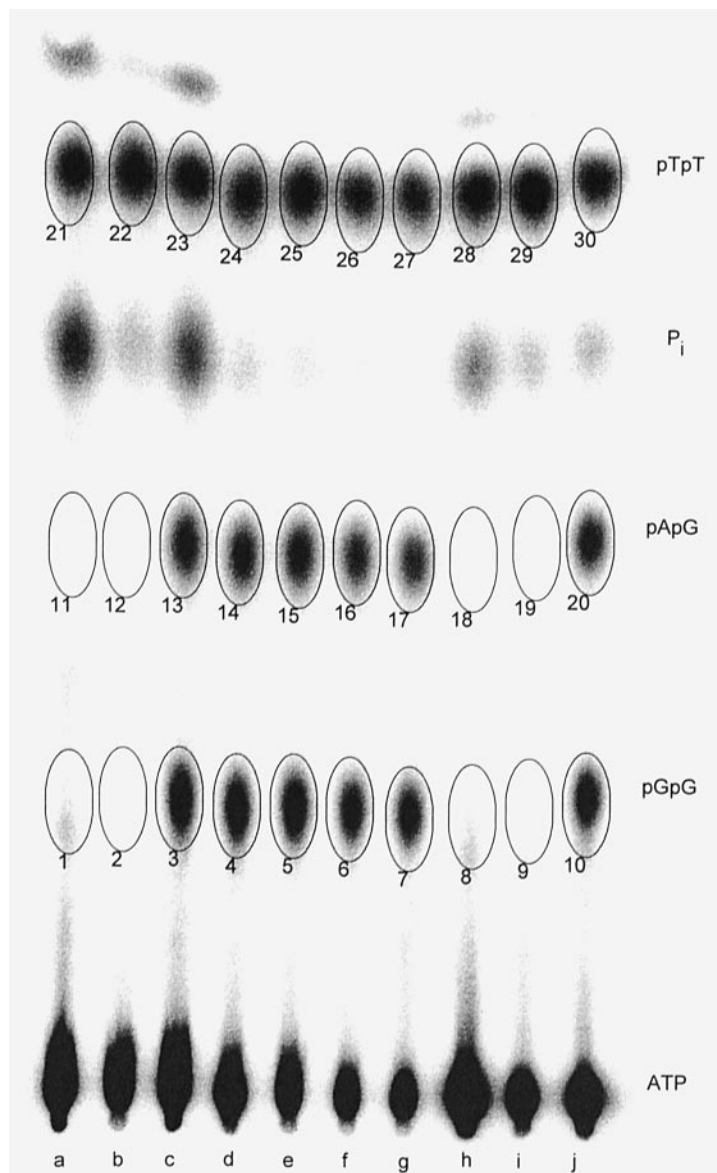
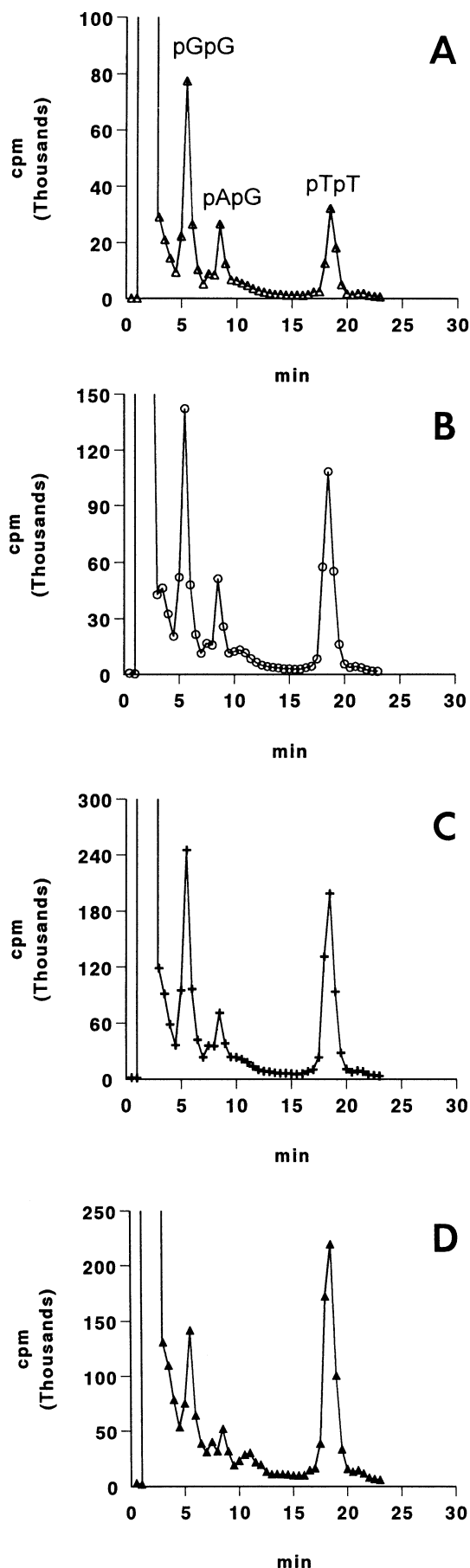


Fig. 1. Phosphorimaging pattern of a TLC sheet after chromatography of ³²P-postlabelled samples, showing the spots of [γ -³²P]ATP, ³²pGpG, ³²pApG, inorganic phosphate (³²P_i) and the internal standard ³²pTpT. Internal standard (500 fmol TpT) was labelled using 2 μ l (lanes a and h) or 0.5 μ l of [γ -³²P]ATP (lanes b and i). Mixtures of 500 fmol of the dinucleotides ApG, GpG and TpT were labelled by use of 2 μ l (lane c and j), 1 μ l (lane d), 0.5 μ l (lane e), 0.4 μ l (lane f) or 0.2 μ l of [γ -³²P]ATP (lane g). The area between ³²pApG and ³²pGpG was taken as background.

Fig. 2. Representative HPLC chromatograms, showing ³²P-labelled pGpG, pApG and the internal standard pTpT as eluted from a C₁₈-column, using 0.1 M KH₂PO₄ (pH 4.0)/2.5% MeOH, 1 ml/min. Fractions of 0.5 ml were collected and counted. The ³²pGpG and ³²pApG originated from the Pt-adducts in DNA isolated from human IGROV-1 ovarian cancer cells incubated with 33.3 (A), 16.67 (B), 8.3 (C) or 3.3 μ M (D) cisplatin for 2 h. The samples injected onto the column were diluted 5.5 times (A), 1.8 times (B) or not diluted (C and D) in order to obtain sufficient high and evaluable peaks.



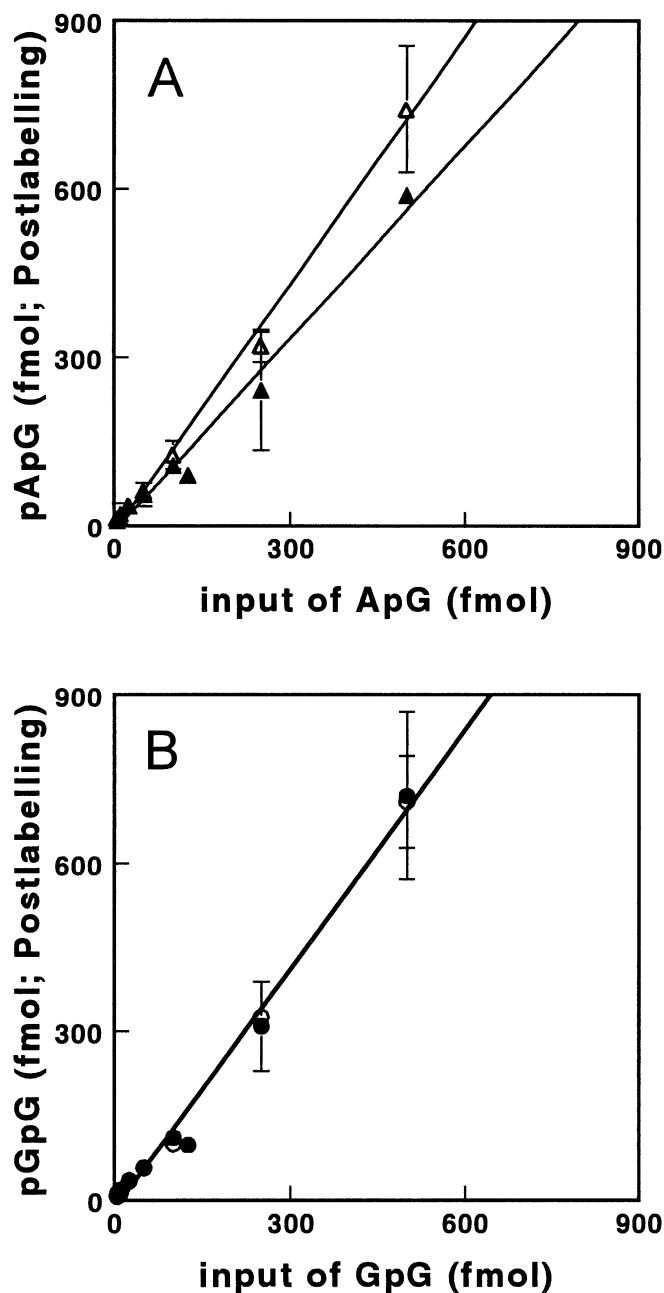


Fig. 3. Relationship between the starting amounts of the dinucleotides (= input) ApG (A) and GpG (B) as determined by weight and their calculated amounts after postlabelling. The postlabelling was performed on the dinucleotides in the presence of the internal standard TpT (open symbols) as well as on mixtures to which 50 fmol of the dinucleotide GpG (A) or ApG (B) was added (closed symbols). Correlation values of 1.0 were found for ApG alone and of 0.99 in combination with 50 fmol GpG (A), of 1.0 for GpG alone and of 0.99 in combination with 50 fmol ApG (B). The dinucleotides ApG and GpG are found to be labelled with equal efficiency. Results are given as means of three independent experiments \pm SD.

responses were obtained for ³²pApG (Figure 3A). R-values of 1.0 were found for ApG alone and of 0.99 in combination with 50 fmol GpG. Similar results were found when variable amounts of GpG were postlabelled (Figure 3B), with R-values of 1.0 for GpG alone and of 1.0 for the combination. These data also indicate that the presence of other dinucleotides hardly interferes with the labelling efficiency of the dinucleotide of interest. Furthermore, the dinucleotides GpG and ApG are shown to be labelled with equal efficiency. These data were

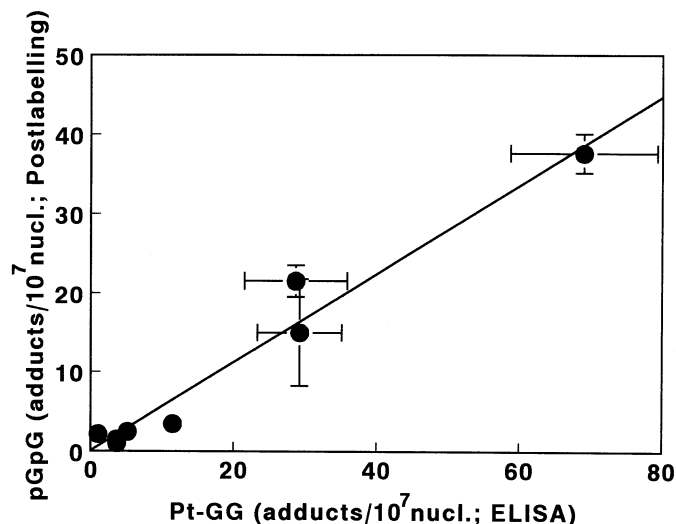


Fig. 4. Correlation between the Pt-GG adduct levels as determined by the ELISA method (using 100 μ g DNA) and the ³²P-postlabelling assay (using 10 μ g DNA) in salmon sperm DNA treated *in vitro* with various concentrations of cisplatin (0 to 42.7 nM) for 5 h at 50°C. The R-value is 0.98 ($P = 0.00003$). Means (adducts/10⁷ nucleotides) of the results are given from two separate experiments (\pm SD).

obtained after a labelling reaction time of 40 min, however, no differences were seen when the reaction was prolonged to 1 h (data not shown). Also when the amount of [γ -³²P]ATP was limited to 0.2 μ l, the substrates were labelled in the same ratio (see Figure 1). When the HPLC method was used for the quantification of the adduct levels in cisplatin-treated ovarian cancer cells, also a linear dose-response was observed (see Figure 2). Comparison of the data obtained with the TLC and HPLC quantification methods indicated that both methods can be used successfully for the determination of small amounts of adducts, i.e. after deplatination and ³²P-postlabelling (data not shown).

Correlation between cisplatin-DNA adduct measurement with the postlabelling assay and the ELISA method

The results after TLC quantification of the postlabelled products were compared with those obtained with the ELISA method of the same DNA samples. For the ELISA method part of the DNA was digested to mononucleotides and 5'-phosphorylated adducts (12). The comparison was made both with *in vitro* cisplatin-treated DNA and with DNA isolated from cisplatin-treated (tumor) tissues. The correlation between the ELISA data and the results obtained after postlabelling of cisplatin-treated salmon sperm DNA, as shown in Figure 4 for the Pt-GG adduct, indicates an R-value of 0.98 ($P = 0.00003$).

In Figure 5 the correlation between the results of the two assays is given for platinated DNA samples isolated from *ex vivo* cisplatin-treated HNSCC as well as from tumor, liver and kidney samples from cisplatin-treated nude mice bearing human ovarian carcinomas. The ELISA data of the latter will be published in detail elsewhere. The corresponding R-values for the Pt-AG and the Pt-GG adducts were 0.77 ($P = 0.045$) and 0.91 ($P = 0.042$) respectively, indicating a good correlation between these two assays.

Correlation of postlabelling results with AAS data

Results obtained with the HPLC-based postlabelling method were compared with those of AAS analysis. For this purpose, part of the DNA isolated from IGROV-1 cells that had

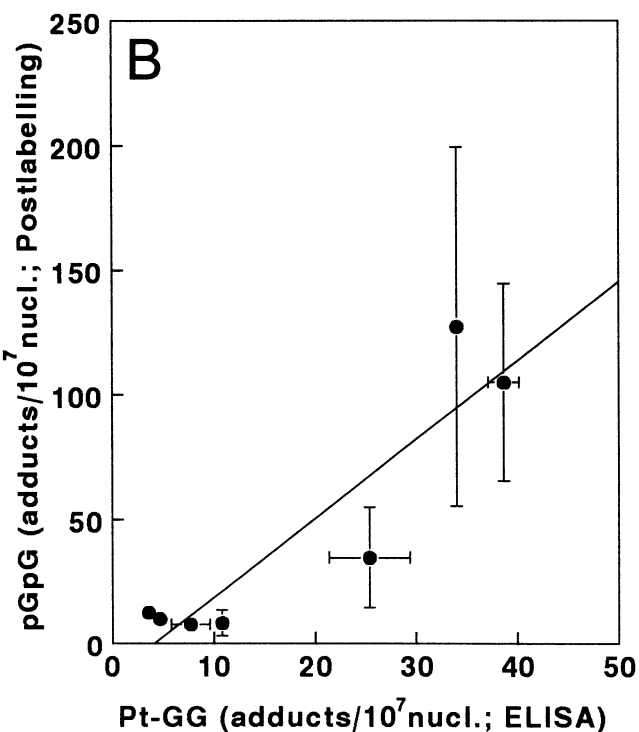
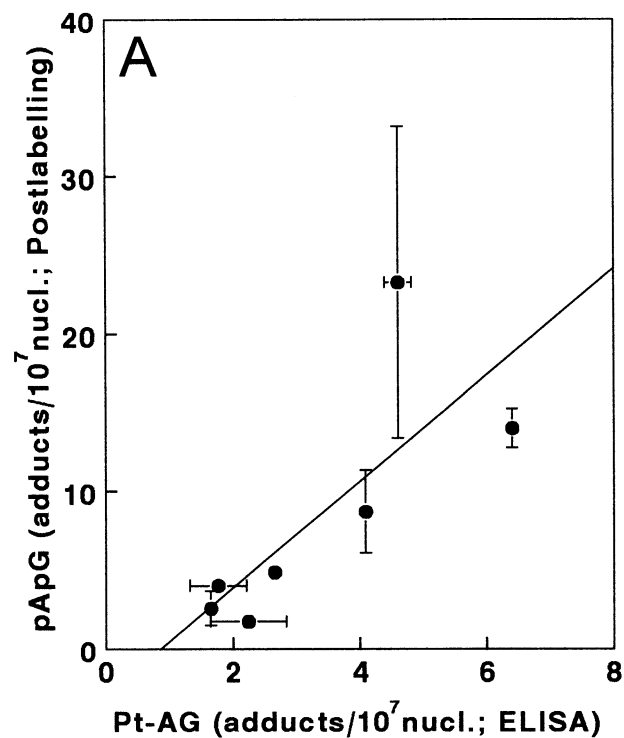


Fig. 5. Correlation between Pt-AG (A) and Pt-GG (B) adduct levels measured with the ELISA method and with the ^{32}P -postlabelling assay (TLC separation) in DNA samples isolated from kidney, liver and xenografts treated with cisplatin either *in vivo* or *ex vivo*. Adduct levels (adducts/ 10^7 nucleotides) for both assays are means \pm SD of data obtained from two independent experiments. The R-value was 0.77 ($P = 0.045$) for Pt-AG and 0.91 ($P = 0.042$) for Pt-GG.

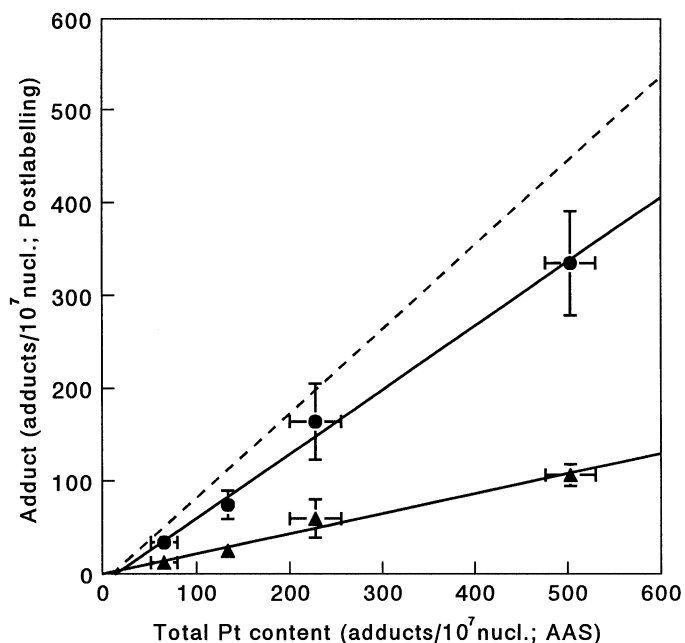


Fig. 6. Correlation between AAS and ^{32}P -postlabelling data of DNA extracts from IGROV-1 ovarian cancer cells treated for 2 h with various concentrations of cisplatin (0–66.7 μM). Part of the DNA was used for determination of the total Pt-content by AAS, whereas the adducts Pt-AG and Pt-GG in the other part were deplatinated, ^{32}P -postlabelled and quantified with the HPLC-based method. Circles, Pt-GG adducts; triangles, Pt-AG adducts; dashed line, total of Pt-GG and Pt-AG adducts. The R-value was 0.99 ($P = 0.012$) for Pt-AG and 1.0 ($P = 0.003$) for Pt-GG. Data (in adducts/ 10^7 nucleotides) are means \pm SD of results from at least three independent experiments.

been incubated with various concentrations of cisplatin, was analyzed by postlabelling and another part by AAS. As shown in Figure 6, highly significant correlations were observed between the total Pt-DNA content (AAS data) and the postlabelling results of the Pt-AG and Pt-GG adducts with R-values of 0.99 ($P = 0.012$) and 1.0 ($P = 0.003$), respectively. The total adduct level measured by postlabelling, i.e. Pt-AG plus Pt-GG, was found to be $\sim 85\%$ of the overall platination level measured by AAS.

Application of the modified postlabelling assay

The assay described here has already been applied successfully for the determination of adduct levels in white blood cells, buccal cells and tumor biopsies from cisplatin-treated cancer patients. The adduct levels in the blood samples, for example, varied between 14 and 22 adducts/ 10^7 nucleotides for the Pt-GG and between 5 and 8 for the Pt-AG adducts after treatment with 70 mg/m^2 cisplatin, whereas in a tumor biopsy 93 Pt-GG and 30 Pt-AG adducts/ 10^7 nucleotides were detected. More data on patient samples will be published elsewhere. The results obtained for the blood cells were in reasonable agreement with those obtained with AAS. In some samples with adduct levels below the detection limit of AAS [~ 8 adducts/ 10^7 nucleotides in minimally 200 μg DNA (5)] some adducts could still be detected by postlabelling [1.6 adducts/ 10^7 nucleotides in 10 μg DNA], which demonstrates the sensitivity of this assay.

Discussion

There are several methods to determine the cisplatin-DNA adduct levels in isolated DNA (3). With the most common of these, AAS, total adduct levels can be determined. A more

sensitive method, in which the various adducts in DNA digests can be measured, was developed by Fichtinger-Schepman and coworkers (12) using competitive ELISA with antisera directed against the specific adducts. However, there is a need for an alternative method to quantify Pt-adducts, because of the limited availability of the antisera, the limitation of the use of the antibodies for only cisplatin and carboplatin adducts and the relatively large amounts of DNA that are required.

Our initial attempt to determine cisplatin–DNA adduct levels was to develop a non-radioactive assay analogous to methods described by Jain and Sharma (21), Kelman *et al.* (22) and Sharma *et al.* (23), using dansylchloride (5-dimethylaminonaphthalene 1-sulphonyl chloride) to label the phosphate groups in adducted nucleotides in order to detect the DNA damage by fluorescence. We tried to dansylate the hydroxyl groups in the deoxyribose moieties under non-aqueous conditions (pyridine) analogous to the binding of *p*-toluenesulphonyl chloride to carbohydrates. A major problem of this approach was the poor solubility of the nucleotides. Another approach was labelling with malondialdehyde, a method first described by Moschel and Leonard (24). Recently, the group of Shuker (25) reported the labelling and detection of various alkyl-guanines by use of phenylmalondialdehyde. In our hands, it appeared to be possible also to label nucleosides in this way, but the method was not sensitive enough to determine low adduct levels as present in biological samples.

In this paper we describe improvements and comparison with conventional methods (ELISA and AAS) of the ³²P-postlabelling assay for the platinated dinucleotides ApG and GpG, as described previously by Blommaert and Saris (1). The most important ameliorations are the addition of an internal standard, a more efficient isolation of the Pt-adducts and a simplification of the deplatination step. Furthermore, for the quantification of the ³²P-labelled products two different methods have been elaborated (see Scheme I for the outline of the whole postlabelling procedure). As the first step in this assay, (isolated) platinated DNA was digested to unmodified deoxynucleosides and platinated adducts. For the dephosphorylation by the enzyme alkaline phosphatase a pH of ~9 is required, which is obtained by addition of only 1/100 volume of 1 M Tris/HCl (pH 9.0) to the digestion mixture instead of the 1/10 volume as reported before (1). This reduced volume is very important in view of the need to decrease the pH to ~3 in order to purify the Pt-adducts on the basis of their positive charge on a cation-exchanger. The higher pH of the DNA digests during this separation step may have been the reason for the rather low recovery of the adducts by these authors (1). The two methods of cation-exchange chromatography presented in this paper, chromatography using the Mono-S and solid-phase extraction on SCX cartridges, are equally effective. An advantage of the Mono-S column is that the amount of DNA applied on the column can be determined directly by comparing the absorbance of the unmodified nucleosides eluted from the column with that of known amounts of digested salmon sperm DNA. During the elution from the cation-exchanger, the Pt-adducts were collected in tubes already containing the dinucleotide TpT as an internal standard and NaCN. The high pH of the resulting solution (~10) prevented digestion of the internal standard by nucleases co-eluted with the Pt-adducts. Another dinucleotide which is not supposed to be present in a platinated DNA digests and therefore a possible candidate for an internal standard is CpC. However, the ³²pCpC product could not be separated

sufficiently from the solvent front during TLC chromatography. Optimal conditions for postlabelling were obtained when the pH of the samples after deplatination was lowered to ~8.2 by addition of NaAc (pH 5.4). Using the approach of small reaction volumes and adjustment of the pH, it appeared not to be necessary to remove the cyanide, which was still prescribed by Blommaert and Saris (1). After the labelling reaction the products were separated by use of either TLC or HPLC (see Figures 1 and 2). With both methods a good separation of ³²pGpG, ³²pApG and ³²pTpT was found, allowing their quantification by phosphorimaging or ³²P-counting. Linearity of the labelling was studied by use of reference dinucleotides ApG and GpG. The results in Figure 3 show a good correlation between the product levels after postlabelling and the starting amounts of the dinucleotides as measured by weight (input). The linearity of the method could be confirmed by analysis of DNA samples isolated from human IGROV-1 ovarian cancer cells treated with various concentrations of cisplatin (Figure 6).

The results of the postlabelling assay were compared with those of the ELISA and with data from total DNA platination levels measured with AAS. Analyses of salmon sperm DNA and of DNA isolated from (tumor) tissues, both *in vitro* treated with cisplatin, as well as of (tumor) tissues treated *in vivo* showed good and significant correlations between postlabelling (with TLC separation) and ELISA data (Figures 4 and 5) for the two adducts Pt-AG and Pt-GG. Although in the same order, the actual quantities of the adducts were different, especially for the isolated DNA samples (Figure 5). These differences, however, are not considered to be significant because in Figure 4 the ELISA data are highest and in Figure 5 the postlabelling data are highest. At least part of the apparent discrepancy can be explained by the variation of the ELISA results. A very good and highly significant correlation was found between the levels of the two adducts in the cellular DNA samples as determined by postlabelling (with HPLC separation) and the total platination levels of the original DNA samples measured with AAS (see Figure 6). The total amount of the two adducts measured by the postlabelling assay, as shown in Figure 6 (dashed line), was found to be ~85% of the overall platination level of the DNA samples, which is in agreement with previous findings (2,26). The same holds, with a few exceptions, for the observed ratio of 3–4 between the Pt-GG and Pt-AG adducts in the cisplatin-treated samples (12,26). Combined with the TLC or HPLC separation technique, this postlabelling assay allows a very sensitive determination of adduct levels, viz as low as 1.6 adducts/10⁷ nucleotides in a 10 µg DNA sample. When larger amounts of DNA are analyzed, even lower adduct levels can be detected. We demonstrated the presence of three adducts per 10⁸ nucleotides, which indicates that the sensitivity of the assay is sufficient for measurements in clinical samples. Because of its high sensitivity, the postlabelling method described in this paper will be very suitable for determining the adduct levels in fine needle aspirates, which often contain not more than 10 µg of DNA. Also adduct levels in DNA isolated from buccal cells have already been determined with this assay. Despite the rather small number of cells that is normally obtained (~3×10⁵), the presence of Pt-GG could be demonstrated in the buccal mucosal cells of a patient after treatment with 70 mg cisplatin/m² (data not shown). In further studies we will give more attention to this application of the ³²P-postlabelling assay for Pt–DNA adducts.

In conclusion, with the modified ³²P-postlabelling assay

described here, the levels of the major Pt-DNA adducts (Pt-AG and Pt-GG) can be measured quantitatively in small quantities of DNA from clinical samples such as white blood cells, (tumor) tissues and buccal mucosal cells. From these results it can be expected that this assay is also applicable for fine needle aspirates. In addition to the determination of cisplatin- or carboplatin-adducts, of which the bifunctional DNA adducts are chemically identical, also adducts on two neighbouring nucleobases formed by other Pt-drugs can be quantified with this assay without further modification. The only prerequisites are that positively charged adducts can be obtained after digestion of the DNA for purification on a cation-exchanger and that the Pt can be removed upon incubation with NaCN.

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