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Liquid chromatographic determination of 2′,3′-dideoxyguanosine in human plasma*

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Abstract: A rapid assay for 2′,3′-dideoxyguanosine (ddG) in human plasma has been developed. The ddG was first extracted from human plasma using solid phase extraction. The extract containing ddG was then assayed with liquid chromatography using an ODS column and a mobile phase of 9% methanol in pH 6.8 phosphate buffer. The overall method had good accuracy (within 2%), linearity (r = 0.9998), sensitivity (LOD = 1.8 ng, S/N = 3) and recovery (>99% for 5–50 μg ddG per ml plasma).

Keywords: *2′,3′-Dideoxyguanosine; plasma; liquid chromatography.*

Introduction

The AIDS epidemic has prompted the search for effective and safe anti-AIDS drugs. Several purine and pyrimidine 2′,3′-dideoxynucleosides including 2′,3′-dideoxyguanosine (ddG) have shown activity against HIV, the AIDS virus [1, 2]. In assessing the activity of a drug, extensive pharmacokinetic and toxicological studies have to be performed. These studies require analytical methods capable of detecting μg ml^{-1} levels of the drug in human plasma.

Liquid chromatography (LC) is, perhaps, the most widely used technique in the analysis of nucleosides in biological fluids. Brown *et al.* [3] have presented an excellent review on the LC analysis of blood nucleosides. The biggest challenge in these analyses is the separation of the analyte nucleoside from endogenous nucleosides and nucleotides. Dideoxynucleosides are more lipophilic than their corresponding deoxynucleosides and nucleosides [4]. Thus, reversed-phase LC appears to be a promising methodology to separate the analyte dideoxynucleosides from the endogenous deoxynucleosides and nucleosides, the latter two being much more polar than dideoxynucleosides. While several methods based on ion exchange [5–7] and reversed-phase [8] LC have been reported for the analysis of 2′,3′-dideoxyadenosine (ddA), 2′,3′-dideoxyinosine (ddI) and 2′,3′-dideoxycytidine (ddC), none has been reported for ddG. In this paper, a simple and rapid assay for ddG in human plasma is presented.

Experimental

Materials

The test compound, ddG (Pharmacia Fine Chemicals), and the LC internal standard, resorcinol (Sigma Chemical Co.), were purchased as pure chemicals (99+%) and used without further purification. Potassium phosphate (Mallinckrodt) was analytical reagent grade, methanol (Mallinckrodt) was ChromAR HPLC grade, and water was HPLC Milli-Q filtered. Human blood, obtained from the Stanford University Blood Bank, was immediately spun down and the plasma collected, divided into 6 ml aliquots and frozen for future use. Sep-Pak C-18 cartridges were purchased from Waters Associates and Bond–Elut disposable columns, each containing 500 mg ODS-bonded silica packing, were obtained from Analytichem International. Solid phase extraction columns were packed in-house with XAD-2 resin (Bio-Rad).

Sample preparation

Standard solutions. A stock solution was prepared by dissolving 5.0 mg ddG in 5.0 ml water. Stock solution dilutions of 1:10, 3:40, 1:20, 1:40 and 1:100 were made with water to give standard solutions of 100, 75, 50, 25 and 10 μg ml^{-1}, respectively.

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Internal standard solution (IS). The IS was prepared by dissolving 15 mg of resorcinol in 25 ml H_2O.

Test solutions. A 1.00 ml aliquot each of the standard solutions was mixed with 1.00 ml of human plasma. A blank solution was also prepared by mixing 1.00 ml of water with 1.00 ml of plasma.

Solid phase extraction (SPE)

Bond–Elut column pretreatment. Each column was washed with 2 ml methanol, followed by 4 ml water, and the residual water was removed with the aid of a vacuum manifold apparatus (Analytichem International).

Elution. Up to 12 pretreated Bond–Elut columns were placed in the vacuum manifold apparatus. Each test solution plus the blank solution was applied to individual columns and eluted at a flow rate of 1 ml min^{-1}. The effluent was discarded. Each column was then rinsed with 2 ml water. After the last trace of water was removed by increasing the vacuum, 2 ml of methanol–water (1:1, v/v) mixture was used to elute the ddG from the column into a 5 ml volumetric flask. A 1.00 ml aliquot of the IS was added and the mixture was diluted to mark with water to form the extract solution.

Liquid chromatography (LC)

The LC system consisted of an IBM LC/9533 solvent delivery system, an IBM LC/9505 automatic sample handler, a Bio-Rad 1305 UV detector, and a Dynamic Solution Maxima data station.

Triplicate 10 μl aliquots of each extract were chromatographed with a DyChrom Chemocopak 5-ODS-H, 150 × 4.6 mm analytical column with a Brownlee NewGuard RP-18 guard column cartridge using a mobile phase of methanol–phosphate (pH 6.8, 0.05 M) (9:91, v/v) at 1.0 ml min^{-1}. Ultraviolet detection was at 254 nm, the absorption maximum of ddG in the mobile phase.

Area or height measurements of the ddG peak, normalized by the IS peak, from each chromatogram of the extracts were compared to those of the respective standard solutions. A 1.00 ml aliquot of each standard solution was mixed with 1.00 ml IS, 2.0 ml methanol–water (1:1, v/v) mixture and diluted to mark with water in a 5 ml volumetric flask before chromatography.

Results and Discussion

To maximize the sensitivity and specificity of an analytical method for biological fluids, the analyte should be concentrated and the interfering substances removed. The strategy used in this assay was to isolate ddG from the plasma with subsequent LC analysis of the isolated material. Because attempts to remove proteins from plasma with ammonium sulphate precipitation led to partial loss of ddG to the precipitate, SPE was used to isolate ddG from human plasma.

Solid phase extraction

The extraction efficiencies of homemade columns of XAD-2 resin, Sep-Pak C_{18} cartridges and Bond–Elut C_{18} disposable columns were evaluated with UV spectroscopy. The extraction recovery of ddG from aqueous solution (100 μg ml^{-1}) using the XAD-2 column was low (<80%). The extract from the Sep-Pak cartridge contained UV-absorbing material that interfered with the evaluation. The Bond–Elut column gave high recovery (>90%) with no interferences and, therefore, was chosen as the support for the SPE. To optimize the recovery, various mixtures of methanol–water were evaluated as the elution solvents. Aqueous solutions with less than 10% methanol were not strong enough to elute ddG from the Bond–Elut column. Although 25% methanol in water would elute ddG, a 50% solution gave consistently high recovery (>98%) in extracting ddG from aqueous solutions and was therefore selected as the elution solvent.

Liquid chromatography

The biggest challenge in an LC analysis of blood nucleosides is to separate the analyte nucleoside from its metabolites and other endogenous nucleosides and nucleotides. The reversed-phase LC method of Kalin and Hill [8] required gradient elution. Using an ODS column, Cheung and Kenney [4] separated ddG from 24 nucleosides and derivatives isocratically. Their reported k' of 15 for ddG, however, was too large for the present purpose. After numerous modifications to Cheung and Kenney's method, an isocratic LC procedure that gave a reasonable k' of 7.5 for ddG was achieved. Figures 1–3 are the chromatograms of ddG standard, SPE extracts of human plasma and a typical test solution (human

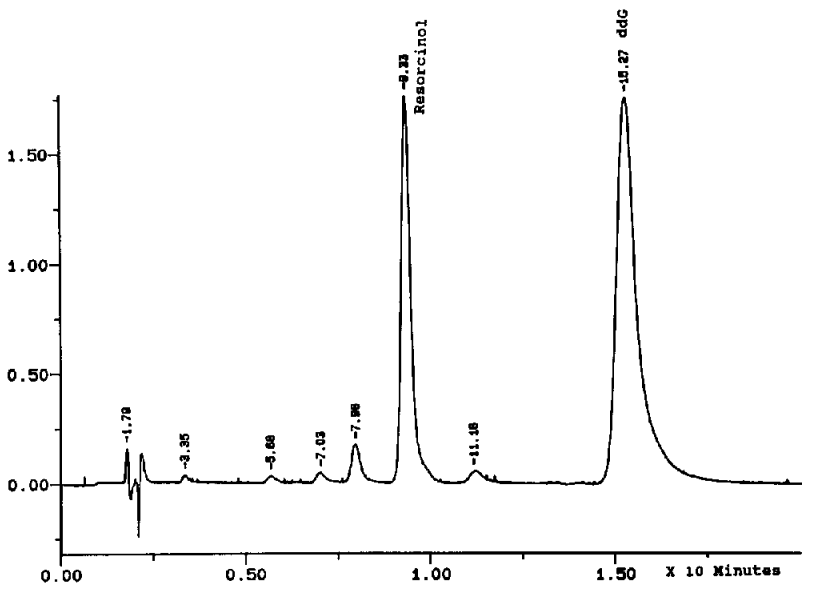

Figure 1
LC chromatogram of a standard solution of ddG in H_2O (100 μg ml^{-1}). Resorcinol, the internal standard, was added to the solution prior to chromatography.

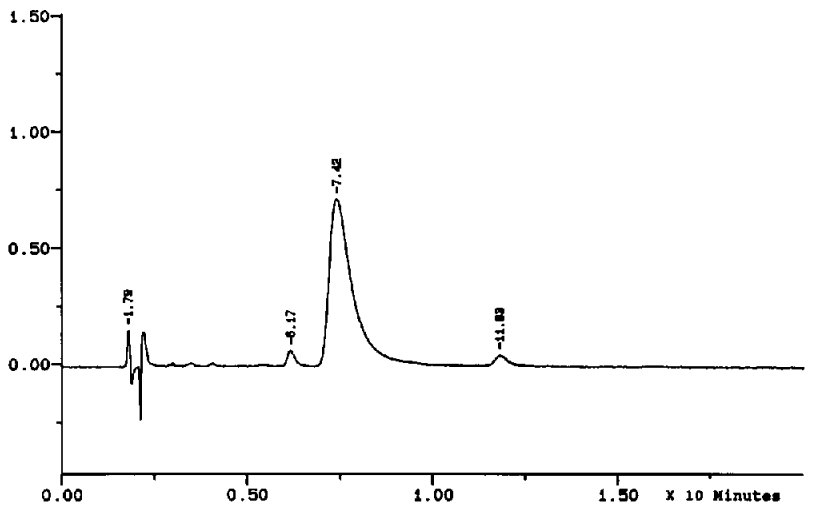

Figure 2
LC chromatogram of a Bond–Elut column extract of human plasma.

plasma spiked with ddG), respectively. It should be noted that the plasma extract showed no component that eluted beyond 20 min ($k' > 10$). The ddG (15.2 min) and the IS (resorcinol, 9.3 min) peaks were verified as homogeneous with a Hewlett-Packard 1040M Photodiode Array Detection system. They were resolved from the plasma background (including endogenous thymidine, adenosine and deoxyadenosine), potential impurities (guanine, guanosine and deoxyguanosine) and metabolites (xanthine and uric acid) of ddG, and other dideoxynucleosides [ddC, ddI and Zidovudine (AZT)] that have shown anti-AIDS activity. Table 1 lists the retention characteristics of the various compounds. The data indicate that the method, although developed specifically for ddG, is potentially applicable to ddC, ddI and AZT.

Special considerations for sample preparation

Several steps in this method deserve comment. Plasma samples were diluted 1:1 with water before the SPE treatment because of the

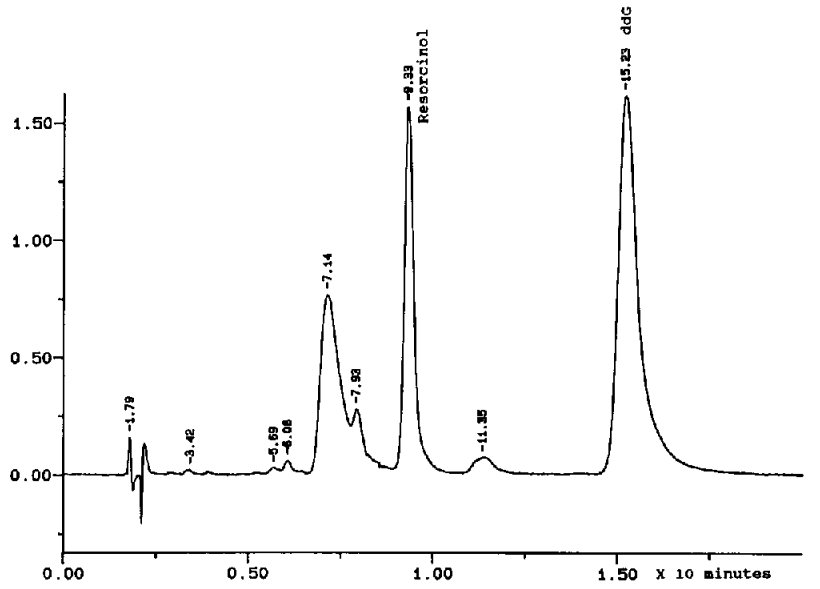

Figure 3
LC chromatogram of a Bond–Elut column extract of a typical test solution (human plasma spiked with ddG at 100 μg ml^{-1}). Resorcinol, the internal standard, was added to the extract prior to chromatography.

Table 1
LC retention times (t_R) and capacity factors (k') of plasma extract components and dideoxynucleosides

Compound	t_R (min)	k'
Unretained	1.8	0.0
Uric acid	2.0	0.1
Guanine	3.4	0.9
Xanthine	3.6	1.0
Hypoxanthine	3.8	1.1
Guanosine	5.8	2.2
Minor component from human plasma	6.1	2.3
2′-Deoxyguanosine (dG)	6.6	2.7
Minor unknown impurity from ddG	7.0	2.9
2′,3′-Dideoxycytidine (ddC)	7.2	3.0
Major component from human plasma	7.3	3.1
Resorcinol (IS)	9.3	4.2
Thymidine (T)	11.0	5.1
Minor unknown impurity from ddG	11.2	5.2
Adenosine (A)	11.6	5.4
Minor component from human plasma	11.8	5.6
2′,3′-Dideoxyinosine (ddI)	12.6	6.0
2′,3′-Dideoxyguanosine (ddG)	15.3	7.5
2′-Deoxyadenosine (dA)	16.3	8.1
AZT	29	15
2′,3′-Dideoxyadenosine (ddA)	>40	>20

LC conditions: DyChrom Chemcopak 5-ODS-H, 150 × 4.6 mm column, isocratic mobile phase of methanol–phosphate (pH 6.8, 0.05 M) (9:91, v/v) at 1.0 ml min^{-1}, and UV detection at 254 nm. Xanthine, hypoxanthine and uric acid were dissolved in water with a small amount of NH_4OH added. The rest of the test compounds were dissolved in water.

high viscosity of some of the plasma samples. Diluting the plasma by 50% with water enhanced the ease of use of the SPE and improved the ddG recovery. When the plasma spiked with ddG was diluted with only a 10% volume of water, the recovery was 93%. Generally, the internal standard (IS) is added to the plasma samples before SPE. The IS should be structurally similar to that of the analyte such that the extraction efficiency for

the analyte and the IS are comparable. This approach is more difficult for the analysis of blood nucleosides because of the presence of endogenous nucleosides which may interfere with subsequent LC analysis. Based on the retention times in Table 1, dA and ddI could be used as the IS in the assay. Unfortunately, dA is an endogenous nucleoside and ddI is a metabolite of ddA. Patients on ddG treatment may also be on ddI or ddA treatment. If ddI is the IS, the method will not be applicable to patients also treated with ddA or ddI. Because of these considerations and the excellent recovery of ddG from SPE of plasma, the internal standard was selected for its chromatographic properties rather than for its structural similarity to the analyte. Consequently, resorcinol was added to the SPE plasma extract prior to the chromatography.

Assay validation

The validity of the LC assay for ddG was demonstrated with chromatographic data derived from ddG standard solutions. The precision based on six injections of a single standard solution (10 μg ddG plus 120 μg IS ml^{-1} water) was within 0.3%. Based on data from the five standard solutions (Table 2), the LC assay was linear ($r = 0.9981$) and accurate (2% error). Based on a 3:1 signal-to-noise ratio, the limit of detection (LOD) for ddG was 1.8 ng.

SPE treatment of the plasma solutions spiked with ddG did not compromise the quality of the assay. Table 3 shows the results of two experiments performed on separate days that used human plasma spiked with various concentrations of ddG. The ddG concentrations in these experiments were 5 to

Table 2
Linearity and accuracy of the LC assay for ddG in water

Sample	Peak intensity ddG	Peak intensity IS	Response factor*	μg ddG per ml water Actual†	μg ddG per ml water Found‡	Error§ %
1	1.505	1.578	0.954	100.0	100.0	0.0
2	0.923	1.290	0.716	75.0	75.3	0.4
3	0.685	1.457	0.470	50.0	49.7	0.6
4	0.304	1.328	0.229	25.0	24.6	1.6
5	0.113	1.241	0.092	10.0	10.4	4.0

See text for LC conditions. Linear regression analysis of response factor (x) vs actual ddG concentration (y) gave $y = 104x + 0.84$, $r = 0.9999$.

*Response factor = (ddG peak)/(IS peak).

†Concentration of the standard solution before addition of IS.

‡Found ddG conc. = 104* (response factor) + 0.84.

§Error = (actual ddG conc. − found ddG conc.)/actual ddG conc.

Table 3
Accuracy of the SPE and LC assay for ddG in human plasma

Experiment	Sample	μg ddG per ml plasma Actual*	μg ddG per ml plasma Found†	Recovery‡ %
1	1	50.0	49.1	98.2
	2	37.5	36.6	97.6
	3	25.0	25.0	100.0
	4	12.5	12.4	99.2
	5	5.0	5.1	102
2	1	50.0	49.3	98.6
	2	37.5	37.1	98.9
	3	25.0	25.5	102.0
	4	12.5	12.6	100.8
	5	5.0	5.0	100

See Experimental for details.

*Actual ddG concentration was the amount of added ddG divided by the volume of the plasma solution before SPE.

†Determined by LC and expressed as the ddG concentration in the plasma solution before SPE.

‡Expressed as found over actual ddG concentration.

50 μg ml^{-1} plasma. The results indicated that the overall method (SPE and LC) was linear (r = 0.9999) and accurate (<2% error). The day to day variation was insignificant and the recovery of ddG by the SPE was >99%.

Potential modifications

The plasma ddG determination presented in this paper has been validated for concentrations of 10 μg ml^{-1} and above. Increasing the LC injection volume to 20 or 100 μl from 10 μl would extend the lower assay limit to 1 μg ml^{-1}. The lower limit could be further extended to 0.3 μg ml^{-1} without modification by reducing the volume of IS added to the 2 ml methanol–water SPE eluate from 33% to 10% and eliminating the dilution with water. The SPE and the LC assay could be modified if further improvement in sensitivity of the method is desired. One modification would be to concentrate the ddG in the SPE extract further by evaporating the methanol from the extract, passing the aqueous residue through another ODS Bond–Elut column, and eluting ddG off the second column with minimal methanol. However, the sensitivity of the method is limited to 2 ng ddG by the UV detection. Although Figs 2 and 3 indicate that the SPE extract of plasma did not interfere with the IS and ddG peaks, at <100 ng ddG per ml plasma level, coextracted endogenous substances have interfered with the LC assay. Examination of the lipophilicities of nucleoside analogues revealed that the partition coefficients (P) of dideoxynucleosides were about 3 and 6 times greater than those of their corresponding deoxynucleosides and nucleosides, respectively [4]. As the P values of deoxycytidine (0.017), deoxyuridine (0.031) and thymidine (0.067) are much less than 3 times that of deoxyguanosine (dG, 0.050) [9], endogenous material of these origins should not interfere with the LC analysis. Since the LC assay presented in this paper was able to separate the endogenous nucleosides A and dA, it is likely that only minor modifications in the LC assay will be needed to adapt the method for plasma ddG determination at <100 ng ml^{-1} levels.

Conclusion

A rapid and simple determination of ddG in human plasma has been developed. The ddG was isolated and concentrated from plasma by SPE with subsequent LC quantitation. The overall method had a recovery of greater than 98% for the range of 5 to 50 μg ddG ml^{-1} plasma. It was specific, linear, accurate (<2% error), and had a sensitivity of 2 ng ddG. Other anti-AIDS dideoxynucleosides such as ddC, ddI and AZT were also separated from the human plasma matrix and from their metabolites with these LC conditions. Therefore, this SPE-LC method is potentially applicable to analyse ddC, ddI and AZT in human plasma.

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