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Determination of leukocyte DNA 6-thioguanine nucleotide levels by high-performance liquid chromatography with fluorescence detection

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Abstract

A HPLC method for determination of 6-thioguanine nucleotide in DNA was developed. Leukocyte DNA was isolated from peripheral blood, derivatized with chloroacetaldehyde and the formed etheno derivatives N^2,3-etheno 6-thioguanine (ε6TG), 1,N^6-etheno adenine (εA) and N^2,3-etheno guanine (εG) were released from the DNA backbone by hydrolysis at pH 6.0 and 80 °C for 60 min. After extraction of ε6TG by immobilized metal ion affinity chromatography (IMAC) the sample was analysed by ion-pair reversed-phase HPLC with fluorescence detection. The limit of quantification was 9.0 nM and the intra- and interday precision ranged from 2.8 to 15.5%. In a small cohort of eight children with acute lymphoblastic leukaemia (ALL), a median of one 6-thioguanine base was found for each 3000 normal bases (range 1:2000–1:11000).

Keywords: 6-Thioguanine nucleotide; Etheno adenine; High-performance liquid chromatography; DNA; Acute lymphoblastic leukaemia; Chloroacetaldehyde; Immobilized metal ion affinity chromatography

1. Introduction

The thiopurines 6-mercaptopurine (6MP) and 6-thioguanine (6TG) have been used since the 1950s for the treatment of haematological malignancies [1,2]. The efficacy of 6MP primarily depends upon a multistep transformation to the active 6-thioguanine nucleotide (deoxy-6-thioguanosine 5′-triphosphate (6TGN)), which is cytotoxic after incorporation into DNA [3]. Owing to large interindividual variations in the metabolism of 6MP the level of 6TGN in erythrocytes (E-6TGN) has been applied to monitor therapy [4–8]. Due to their high number in peripheral blood, erythrocytes are convenient for this purpose, but little is known of the correlation between E-6TGN and the incorporation of 6TGN into leukocyte DNA (DNA–6TGN) and the relation of DNA–6TGN to treatment efficacy.

Several methods utilizing HPLC for determination of total 6TGNs (6TG, 6-thioguanosine 5′-monophosphate, 6-thioguanosine 5′-diphosphate and deoxy-6-thioguanosine 5′-triphosphate) in erythrocytes have been published [9–16]. However, none of these methods are sensitive enough to allow determination of incorporation of 6TGN into DNA in small aliquots of blood (1–2 ml). Although methods for quantification of DNA–6TGN have been described previously [17–19], these methods have not been used for larger studies of the influence of incorporation of 6TGN into DNA in clinical trials. One method is based on enzymatic hydrolysis of DNA to nucleotides followed by oxidation with alkaline potassium permanganate and quantification of a fluorescent oxidation product of 6TGN by HPLC [17]. Warren et al. described two other methods based on enzymatic hydrolysis of DNA to nucleosides, derivatization of 6-thioguanine nucleoside with the thiol-reactive fluorophores monobromobimane [18] or *N*-[6-(7-amino-4-methylcoumarin-3-acetamido)hexyl]-3′-(2′-pyridyldithio)propionamide (AMCA-HPDP) [19] and quantification of the derivatives by HPLC with fluorescence detection. Since we could not reproduce the results of these assays, we developed a new method for the determination of DNA–6TGN. The procedure involves derivatization with

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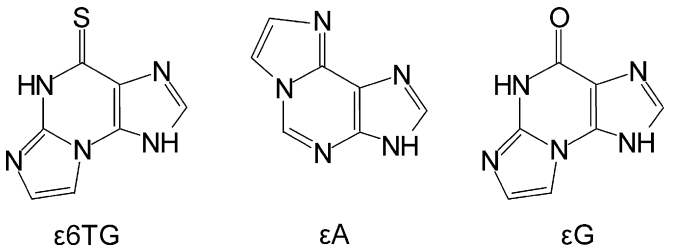

Fig. 1. Chemical structures of the etheno derivatives N^2,3-etheno 6-thioguanine (ε6TG), 1,N^6-etheno adenine (εA) and N^2,3-etheno guanine (εG).

chloroacetaldehyde (CAA) and hydrolysis of the formed etheno derivatives: the proposed N^2,3-etheno 6-thioguanine (ε6TG), 1,N^6-etheno adenine (εA) [20] and N^2,3-etheno guanine (εG) [21] (Fig. 1) from DNA, extraction of ε6TG by immobilized metal ion affinity chromatography (IMAC) and quantification by ion-pair reversed-phase HPLC with fluorescence detection. A reversed-phase HPLC method with UV detection quantifies εA in order to determine the 6TG/adenine ratio and hence, the level of 6TG residues incorporated into leukocyte DNA.

2. Materials and methods

2.1. Chemicals and reagents

6-Thioguanine (6TG), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) and 2-(4-morpholino) ethanesulfonic acid sodium salt (MES) were obtained from Sigma (Sigma–Aldrich, St. Louis, MO, USA). Adenine was from Applichem (Darmstadt, Germany). Human leukocyte DNA was obtained from healthy adult donors. Chloroacetaldehyde (CAA, 50 wt.% solution corresponding to 8 M) and phosphoric acid were from Aldrich (Sigma–Aldrich Chemie, Steinheim, Germany). 5-Acetaminosalicylic acid was a gift from Ferring A/S (Copenhagen, Denmark). Ethanol was from V&S Distillers (Aalborg, Denmark). Di-sodium tetraborate decahydrate was from Ferak Laborat Gmbh (Berlin, Germany) and heptafluoro butyric acid (HFBA) from Fluka (Buchs, Switzerland). Ammonium acetate, copper(II) sulphate pentahydrate, glacial acetic acid, hydrochloric acid, potassium di-hydrogen phosphate, potassium hydroxide, 2-propanol and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Ammonium formate, sodium acetate and sodium chloride were obtained from Riedel-de-Haën (Sigma–Aldrich Laborchemikalien, Seelze, Germany). Ethylenediaminetetraacetic acid (EDTA), methanol and Triton X-100 were from BDH (VWR International, Poole, England). Sucrose was from Acros Organics (Geel, Belgium), proteinase K was from Roche Diagnostic (Basel, Switzerland) and 1 M Tris–HCl, pH 7.5 was from Invitrogen (Carlsbad, CA, USA). 1 M magnesium chloride was purchased from Region H Apoteket (Copenhagen, Denmark) and sodium dodecyl sulphate was from Bie & Berntsen (Rødovre, Denmark). Chelating Sepharose Fast Flow and blue dextran 2000 were from Amersham Pharmacia Biotech (Uppsala, Sweden).

All chemicals were of analytical grade or HPLC quality. Purified water from a Milli-Q deionization unit (Millipore, Bedford, MA, USA) was used throughout.

The potassium phosphate buffers at pH 6.0 were prepared by dissolving potassium di-hydrogen phosphate and pH was adjusted using 5 M potassium hydroxide. In addition, the following buffers and solutions were prepared: *derivatization reagent*, 1 M potassium phosphate buffer (pH 6.0):CAA (1:1 v/v); *EDTA solution*, 0.1 M EDTA and 0.1 M MES; *eluting* and *washing solution*, 0.05 M phosphoric acid and 0.5 M sodium chloride adjusted to pH 2.5 using 5 M potassium hydroxide; *equilibration solution*, 0.05 M potassium phosphate buffer (pH 6.0) containing 0.5 M sodium chloride.

2.2. Equipment

The chromatographic equipment for determination of ε6TG consisted of an Agilent 1100 Series LC system (Agilent Technologies, Palo Alto, CA, USA) with a binary pump (G1376A), a vacuum degasser (G1379A), an autosampler (G1313A), a thermostated column compartment (G1316A) and a fluorescence detector (G1321A). For determination of εA we applied an isocratic pump (G1310A) and an ultraviolet detector (G1365A). An Agilent Chemstation software package (Rev. A.08.03) was used for instrument control, data acquisition and data handling in both systems. Separations were carried out on two identical columns, one for each HPLC system. The columns were Luna C18(2) columns (100 mm × 2.0 mm i.d., particle size 3.5 μm) protected by SecurityGuard columns containing the same column packing material from Phenomenex (Torrance, CA, USA).

2.3. Chromatographic conditions

2.3.1. Determination of etheno 6-thioguanine—HPLC system I

The mobile phase consisted of water:methanol (95:5 v/v) with 0.5% HFBA. Separation was performed under isocratic conditions at a flow rate of 0.3 ml/min in 30 min. Column temperature was maintained at 40 °C, and excitation and emission wavelengths were set at 305 and 445 nm, respectively. Seventy microlitres of the final sample preparation was injected.

2.3.2. Determination of etheno adenine—HPLC system II

The mobile phase was composed of 0.1 M potassium phosphate buffer (pH 6.0):methanol (9:1 v/v) and delivered at a flow rate of 0.2 ml/min. Analysis time was 9 min. Column temperature was fixed at 45 °C. The ultraviolet detector was operated at a wavelength of 275 nm. Fifty microlitres was injected.

2.4. Sample preparation

2.4.1. DNA isolation

DNA was prepared by addition of 1 ml buffer (0.32 mM sucrose, 5 mM magnesium chloride, 1% Triton-X and 1 mM Tris–HCl at pH 7.5) to 1 ml of whole blood. After centrifugation (5244 × *g* for 15 min) at 21 °C the nuclear pellet was resuspended in 750 μl of a solution containing 75 mM sodium

chloride and 24 mM EDTA (pH 8.0) and was disrupted by the addition of 20 μl 20% (w/v) sodium dodecyl sulphate. The mixture was incubated at 56 °C with proteinase K (500 μg/ml) until the pellets were dissolved (1–24 h). After precipitation of the proteins by addition of 300 μl saturated sodium chloride, DNA was extracted with 2-propanol. DNA was precipitated by the addition of 1 ml ethanol (70% v/v). The sample was dried at 65 °C for 10–20 min. The DNA was resuspended in 400 μl water and stored at −80 °C.

Suspensions of DNA from healthy adult donors were isolated as described for the preparation of patient DNA suspension. DNA was isolated from 15 ml of whole blood and resuspended in 750 μl water.

2.4.2. Calibration standards

30.0 μM stock solution: 50 mg 6TG was dissolved in 600 μl 1 M sodium hydroxide and diluted with water to 10.0 ml. Working standard solutions (11.4, 37.7, 62.9, 89.1, 114.3 and 140.6 nM) were prepared by appropriate dilution of the stock solution. Calibration standards were prepared in duplicate by spiking 50 μl of a DNA suspension from a healthy adult donor with 350 μl of each working standard solution of 6TG to obtain calibration standard samples (10.0, 33.0, 55.0, 78.0, 100.0 and 123.0 nM).

A stock solution of adenine at a concentration of 37.0 mM was prepared by dissolving 50 mg in 2 ml 5 M hydrochloric acid followed by heating. After cooling the solution was further diluted in water to 10.0 ml. Calibration standards (10.0, 30.0, 45.0, 65.0, 80.0 and 100.0 μM) were prepared in duplicate by appropriate dilution of the stock solution.

2.4.3. Derivatization and hydrolysis

Fifty microlitres *derivatization reagent* was added to 400 μl of sample (either patient DNA suspension, calibration standard of 6TG or calibration standard of adenine) and the mixture was incubated at 80 °C for 60 min. Immediately after derivatization, 20 μl of the εA mixture was diluted into 180 μl 0.1 M potassium phosphate buffer (pH 6.0) and 50 μl of this solution was injected onto HPLC system II. The remaining sample from the patient DNA suspension sample or the calibration standard of 6TG was subjected to extraction by immobilized metal ion affinity chromatography (IMAC) in order to determine ε6TG.

2.4.4. Immobilized metal ion affinity chromatography (IMAC)

One microlitre solid phase extraction cartridges (Waters Corporation, Milford, MASS, USA) were packed wet, as described by Bendahl et al. [22], with Chelating Sepharose Fast Flow to give a final bed height of 17 mm, corresponding to 250 μl sorbent. Using blue dextran 2000, the dead volume of the packed cartridges was quantified to 110 μl.

The sorbent was conditioned using the following steps: 1250 μl water, 100 μl 0.1 M copper(II) sulphate, 1250 μl water, 1250 μl *washing solution* and finally 1250 μl *equilibration solution*. The sorbent was regenerated by adding 1250 μl *EDTA solution* followed by 1 M sodium hydroxide and 0.01 M sodium hydroxide and was finally stored in ethanol (20% v/v). The cartridges were run at gravitational flow velocity and allowed to drain until the flow stopped between each operation. Drops hanging from the tip of the cartridge outlet at the end of the washing and elution procedures were adhered to the receiver vial by touching the inside wall of the vial.

The samples were treated in the following way: 75–80 mg MES and 25–30 mg sodium chloride were added to each of the remaining sample from the patient DNA suspension sample or the calibration standard of 6TG sample. Four hundred twenty-five microlitres sample was transferred to the previously conditioned sorbent in solid phase extraction cartridges. After loading, the sorbent was washed with 500 μl *equilibration solution* and 250 μl *eluting solution*. The ε6TG fraction was eluted with 200 μl *eluting solution* into a sample vial containing 20 μl 10 μM 5-acetaminosalicylic acid (volume marker) and mixed thoroughly. Seventy microlitres was injected onto HPLC system I. The procedure is summarized in Fig. 2.

2.5. Method validation

The analytical methods, including the hydrolysis, derivatization, extraction and HPLC analysis, were validated in terms of linearity, limit of quantification (LOQ), limit of detection (LOD), intra- and interday precision and accuracy and recovery.

LOQ was calculated as 10× standard deviation/concentration$_{nominal}$ at the lowest concentration level and LOD was calculated as 3× standard deviation/concentration$_{nominal}$ at the lowest concentration level (10.0 nM for 6TG and 10.0 μM for adenine). The intraday precision was determined at three concentration levels (10.0, 55.0 and 100.0 nM 6TG and 10.0, 45.0 and 80.0 μM adenine) using six replicates within the same day and then calculating the coefficient of variation

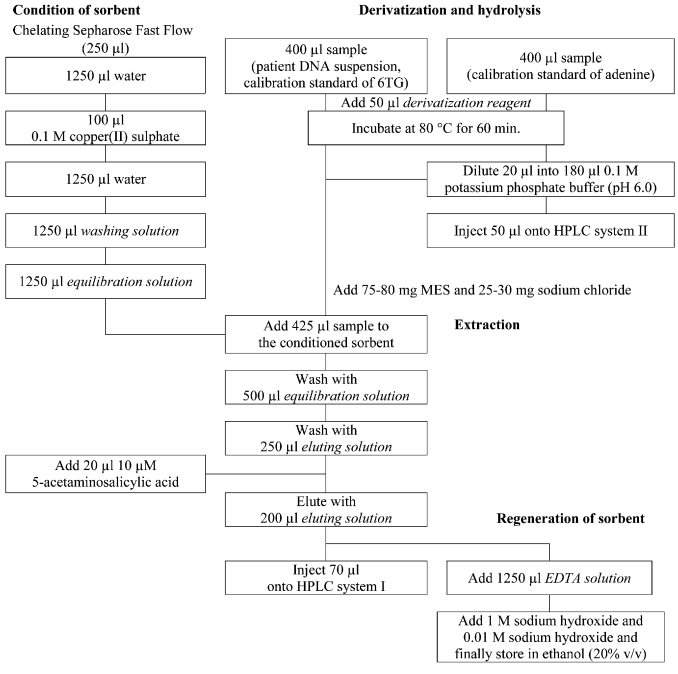

Fig. 2. Procedure for derivatization, hydrolysis and extraction of samples for determination of DNA–6TGN.

(CV). Accuracy was measured as the %difference from nominal concentration (accuracy (%) = (concentration$_{measured}$/concentration$_{nominal}$) × 100). Interday precision and accuracy were calculated by analysing the three concentration levels in duplicate within 3 consecutive days. The recovery of ε6TG from the IMAC extraction procedure was determined by comparing the measured concentration from an extracted sample with an unextracted sample. The measured concentration of εA was converted to an amount of DNA by the knowledge that 29.3% of bases in human DNA are adenine and compared to the amount of DNA determined by the routinely used spectrophotometric method where the absorbance (Abs) of a solution containing DNA is measured at UV 260 nm and the amount of DNA is measured as Abs × 50 μg DNA/ml [23].

3. Results and discussion

A new method for the determination of DNA–6TGN was developed as the methods described by Warren et al. [18,19] did not provide reproducible results. It was not possible to obtain a fluorescent derivative by reaction of 6TG with monobromobimane [18], and non-reproducible results were obtained by analysis of DNA from patients according to the assay involving derivatization of 6TG with *N*-[6-(7-amino-4-methylcoumarin-3-acetamido)hexyl]-3′-(2′-pyridyldithio)propionamide (AMCA-HPDP) [19].

3.1. Derivatization and hydrolysis

The effect of pH and type of buffer substance on the derivatization process of 6TG at 80 °C for 60 min was investigated. The tested buffer substances were ammonium acetate, Britton–Robinson (acetate–borate–phosphate), potassium phosphate and sodium acetate and in the pH range 2.0–12.0. In this pH-range the potassium phosphate buffer was superior and gave a high and constant response in the pH range 3.0–6.0.

In the literature, derivatization of adenine at 80 °C between 15 and 40 min in phosphate buffer at pH 6.0–7.0 has been reported [24,25]. To investigate simultaneous the reaction time for both derivatization of DNA and hydrolysis of the formed derivatives from the DNA backbone, a suspension of DNA from a healthy adult donor was derivatized with 0.4 M CAA in 0.05 M potassium phosphate buffer (pH 6.0) at 80 °C for 10–120 min and compared with the reaction time for derivatization of adenine. The reaction time for derivatization of 6TG was studied at similar conditions in the time range 5–120 min.

Fig. 3 illustrates that a maximum yield of ε6TG is obtained in 30 min. After 40 min a maximum of εA is reached where as the formation and release of εA from DNA needs 80 min to reach maximum showing that the hydrolysis requires about 40 min to complete.

3.2. Extraction

In order to obtain reliable quantitative data on ε6TG it was necessary to remove most of the εA before the HPLC analysis. Therefore, extraction by immobilized metal ion affinity chromatography (IMAC) with subsequent selective elution of ε6TG was performed. Due to the relative high intensity of the fluorescence signal of εA and the huge amount of adenine in DNA compared to 6TG, a sufficient chromatographic separation of ε6TG and εA was not possible without the IMAC extraction.

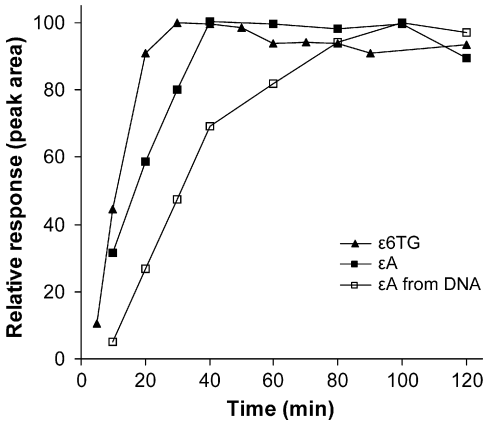

Fig. 3. Reaction time for derivatization of 6TG and adenine to form ε6TG and εA, respectively, and derivatization and hydrolysis of DNA to form εA at 80 °C. A 100 nM 6TG solution, a 60 μM adenine solution (corresponding to maximum adenine concentration obtained from the suspension of DNA from a healthy adult donor) and a suspension of DNA from a healthy adult donor in 0.05 M potassium phosphate buffer (pH 6.0) and 0.4 M CAA were used. Measured by HPLC with fluorescence detection.

The adsorption in IMAC is mediated by the interaction of aromatic nitrogens in imidazole with the chelated metal ion [26]. Because the etheno derivatives share this imidazole moiety, the compounds are adsorbed by the sorbent. The influence of different buffer substances and pH values on the adsorption and elution of ε6TG and εA have been studied. Adsorption of ε6TG and εA was independent of the tested buffer substances ammonium acetate, HEPES, MES and potassium phosphate, all added ammonium formate or sodium chloride to avoid unwanted ion exchange effects [27]. But the pH strongly influenced the adsorption on the sorbent during washing with the different equilibration solutions. The etheno derivatives were retained on the sorbent at pH 6.0–6.5 but the etheno derivatives were less strongly bound to the sorbent when equilibration solutions with pH 7.5–8.0 were used (Fig. 4a). By controlling pH at pH 2.5 in the eluting solution it is possible to selectively elute ε6TG with only a minor elution of the adsorbed εA (Fig. 4b).

An elution profile was generated by collecting small fractions from the cartridges. A calibration standard at 100 nM 6TG was derivatized, hydrolysed and extracted as described in Section 2.4. After loading of the sample onto the sorbent, the sorbent was added five times 100 μl *equilibration solution* followed by 12 × 50 μl *eluting solution.* Each fraction of the eluate from the cartridges was analysed by injection of 40 μl onto HPLC system I. The elution profile presented in Fig. 5 shows that about 10% of ε6TG eluted during sorption and washing with the *equilibration solution*. The remaining ε6TG was bound strongly to the sorbent and eluted with the *eluting solution* by lowering the pH from 6.0 to 2.5. When preparing samples in the final method the first 11 fractions (eluate during loading of sample, 500 μl *equilibration solution* and 250 μl *eluting solution*) were colleted as one pool

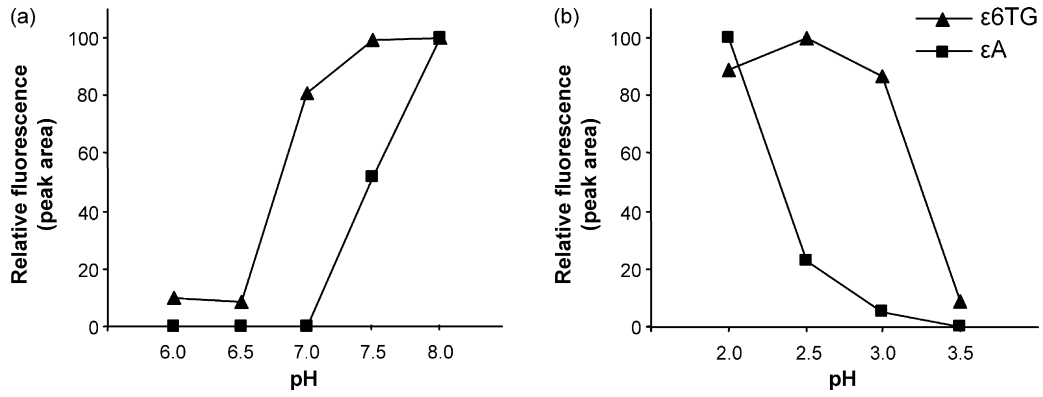

Fig. 4. Effect of pH in the equilibration solution and eluting solution on adsorption (a) and elution (b) of ε6TG and εA from the conditioned sorbent. Measured by HPLC with fluorescence detection. Equilibration solutions: 0.02 M MES, 1 M ammonium formate buffers (pH 6.0 and 6.5) and 0.02 M HEPES, 1 M ammonium formate buffers (pH 7.0, 7.5 and 8.0); eluting solutions: 0.05 M potassium phosphate, 0.5 M sodium chloride buffers (pH 2.0, 2.5, 3.0 and 3.5).

and discarded, while fraction 12-15 (in total corresponding to 200 μl *eluting solution*) were colleted as one pool. Finally, 20 μl 10 μM 5-acetaminosalicylic acid was added to the eluate as a volume marker for correction of differences in eluted volume from each cartridge. It has not been possible to find a true internal standard that could be added at the hydrolysis step. When analysing εA the only further sample preparation after derivatization was a single dilution. Hence, no internal standard was used.

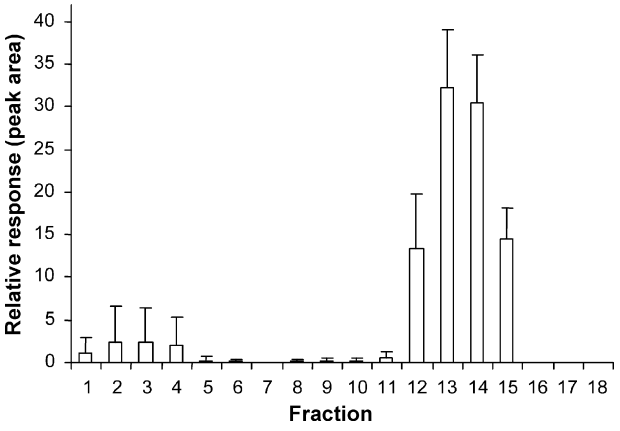

Fig. 5. Elution profiles obtained from three different solid phase extraction cartridges (mean ± SD) each packed with Chelating Sepharose Fast Flow (250 μl sorbent) and applied a derivatized and hydrolysed calibration standard of 100 nM 6TG after condition of the sorbent. Fraction 1 represents the eluate during loading of sample. Fraction 2–6 the eluate during addition of 100 μl *equilibration solution* each and fraction 7–18 represent the eluate from the added *eluting solution*, 50 μl each. Measured by HPLC with fluorescence detection.

3.3. Validation

The two analytical procedures, including the hydrolysis, derivatization, extraction and HPLC analysis, for the assay of 6TG and for adenine by HPLC were validated with respect to linearity, LOQ, LOD, intra- and interday precision and accuracy and recovery. The regression line for 6TG (determined as ε6TG) was calculated as $y = a + bx$, where x was the 6TG concentration (nM) and y was the fluorescence response (peak area of ε6TG/peak area of volume marker). The regression line for adenine (determined as εA) was calculated in the same manner, where x was the adenine concentration (μM) and y was the UV response (peak area of εA). The representative linear equation was $y = (-0.01 \pm 0.03) + (0.0236 \pm 0.0007)x$ for the determination of 6TG and for the determination of adenine the linear equation was $y = (5 \pm 1) + (7.9 \pm 0.2)x$. The correlation coefficient (r) values were 0.992 (±0.006) and 0.9995 (±0.0002), respectively. For 6TG LOQ was 9.0 nM and LOD 2.7 nM. LOQ and LOD for adenine were 3.6 μM and 1.1 μM using UV detection, respectively. The intra- and interday precision and accuracy of the standards are listed in Table 1. The values of the preci-

Table 1
The intra- and interday precision and accuracy

Nominal concentration	Intraday		Interday	
	Precision (CV) ($n = 6$)	Accuracy (%)[a] ($n = 6$)	Precision (CV) ($n = 10$)	Accuracy (%)[a] ($n = 10$)
6TG (nM)				
10.0	10.8	86.4	15.5	92.9
55.0	2.8	103.0	4.7	104.7
100.0	8.2	99.5	8.5	101.8
Adenine (μM)				
10.0	3.8	98.0	3.1	98.1
45.0	2.6	100.0	2.2	99.7
80.0	2.4	100.2	1.9	100.4

[a] Accuracy (%) = (concentration$_{measured}$/concentration$_{nominal}$) × 100.

Table 2
Results from eight children on 6MP maintenance treatment for ALL

Patient	DNA–6TGN		E-6TGN
	pmol 6TG/μmol adenine	6TG base:total bases[a]	nmol 6TG/mmol haemoglobin[b]
A	306	1:11000	93
B	1145	1:3000	215
C	1491	1:2500	188
D	484	1:7000	127
E	770	1:4500	397
F	1972	1:2000	682
G	1128	1:3000	233
H	1330	1:2500	126

Data are means of two individually prepared samples from the same patient.
[a] Number of 6TG bases compared to the total number of normal bases. The total number of normal bases was calculated as mole adenine × 100/29.3. In humans, 29.3% of bases in DNA are adenine [23].
[b] See Ref. [9].

sion are in all cases better than 15.5% and 3.8% CV for 6TG and adenine, respectively. For 6TG the intraday accuracy ranges from 86.4 to 103.0% and the interday accuracy varies from 92.9 to 104.7%. The intra- and interday accuracy for adenine ranges from 98.0 to 100.2% and 98.1 to 100.4%, respectively. All the intra- and interday precision and accuracy values are considered acceptable for the intended purpose. The recovery of ε6TG from the IMAC extraction procedure was 90% and the amount of εA corresponded to 83% of DNA found by the spectrophotometric method. The total recovery of ε6TG and εA is estimated to 75%.

The stability of ε6TG and εA in the autosampler at ambient temperature were investigated. The prepared ε6TG solutions with the concentrations 10.0, 55.0 and 100.0 nM 6TG were injected three times during 72 h and 97–101% was recovered. After storing in the autosampler for 52 h 95–98% of εA at the concentrations 30.0, 80.0 and 100.0 μM adenine was found. Thus, the samples are considered stable for this time period.

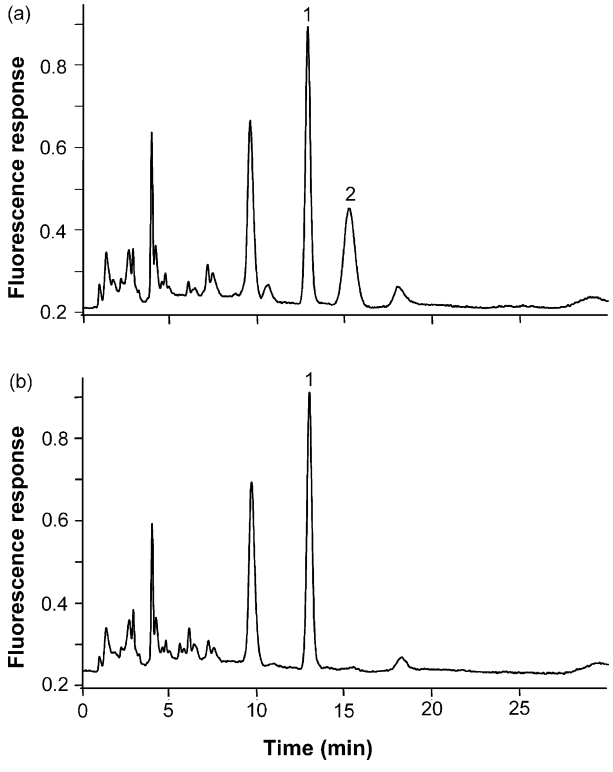

Fig. 6. Chromatograms of a sample from a patient under 6MP therapy (a) and a suspension of DNA from a healthy adult donor (b). 1,5-Acetaminosalicylic acid (volume marker); 2, ε6TG. The area of peak 2 corresponds to 33.6 nM 6TG in whole blood from a patient under 6MP therapy. HPLC system I, column: Luna C18(2) (100 mm × 2.0 mm i.d., particle size 3.5 μm), temperature: 40 °C, mobile phase: water:methanol (95:5 v/v) with 0.5% HFBA, isocratic, flow: 0.3 ml/min, injection volume: 70 μl, fluorescence detection: excitation 305 nm, emission 445 nm.

3.4. Analysis of patient samples

The method developed was applied for the determination of the incorporated 6TG in leukocyte DNA in 1 ml blood samples from a small cohort of patients on 6MP therapy for acute lymphoblastic leukaemia (ALL) (Table 2). The levels of 6TG residues incorporated were found to vary over a 6-fold range from 306 to 1972 pmol 6TG/μmol adenine corresponding to an incorporation of one 6TG for every 2000–11000 normal bases. This is in the same range found by Warren et al. [19]. The levels of E-6TGN [9] varied from 93 to 682 nmol 6TG/mmol haemoglobin (Table 2).

Typical chromatograms from HPLC system II of a sample from a patient who received 6MP and a sample from a healthy adult donor are shown in Fig. 6.

4. Conclusion

This new ion-pair reversed-phase HPLC method with fluorescence detection offers a precise and reliable method for the determination of 6TGN in leukocyte DNA.

The method was applied for determination of the incorporation of 6TGN into DNA in small aliquots of blood, which is crucial for clinical studies in children. No linear relationship between E-6TGN and DNA–6TGN was found. Larger clinical studies are needed to clarify the applicability of the pharmacological parameter for toxicity and response prediction as well as dose adjustments in patients on thiopurine therapy.

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