Pergamon

0731-7085(94)00102-2

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 12, pp. 1495–1499, 1994
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0731-7085/94 $7.00 + 0.00

Design and applications of a new fluorimetric assay of thioguanine in liposomes

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Abstract: This paper describes the systematic design of a modification that overcomes the difficulties encountered with the original fluorimetric method for thioguanine. It allows the determination of thioguanine alone, as well as in a lipid medium (specifically in liposomes). It consists of an earlier lipid extraction based on liquid–solid extraction cartridges; then the oxidation of thioguanine in the lipid-free solution is performed by adding hydrogen peroxide in pH 4.7 acetate buffer at 50°C for 30 min. A central composite design was used to finally optimize the method. The assay is precise (RSD values were 1.8 and 2.1% for intra-day and between-day precision, respectively). The detection limit was 0.05 μM and the limit of quantitation 0.08 μM.

Keywords: *Thioguanine; fluorescence spectrophotometry; liposomes; solid–liquid extraction; central composite design.*

Introduction

6-Thioguanine (2-amino-1,7-dihydro-6-thiopurine) (Fig. 1) is a chemotherapeutic drug used as an immunosuppressive agent and also for the treatment in the advanced stages of leukemia. As with other chemotherapeutic drugs, namely doxorubicin, vincristine, vinblastine, cis-platinum, methotrexate, etc. it has been encapsulated in liposomes in order to check whether there are differences between the therapeutic action of the free drug and its liposomal form, as well as to check any differences in their adverse effects, mainly the hepatic and haematological effects. Since the encapsulation efficiencies obtained, that is, the relationship between the amount of drug in liposomes and the amount of lipid present, are relatively low, very sensitive and specific assays are needed to determine the concentration of the thioguanine in this lipid system. However, very few methods have been reported for such determinations.

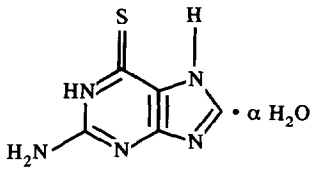

Figure 1
Structure of thioguanine.

The simplest method of analysis is the spectrophotometric procedure: thioguanine absorbs UV radiation, its absorption pattern being pH-dependent: at pH 1 the maximum absorption (λ_{max}) is at 347 nm with a molar absorptivity (ϵ) of 20,425 M^{-1} cm^{-1}; at pH 4.7, λ_{max} = 342 nm, ϵ = 18,500 M^{-1} cm^{-1}, and at pH 9.3, λ_{max} = 324 nm, ϵ = 14,800 M^{-1} cm^{-1}. Unfortunately, the sensitivity of this procedure falls far short of requirements.

Spectrofluorometric methods of analysis are, generally, more sensitive than spectrophotometric methods. Thioguanine is only weakly fluorescent, but it is possible to increase the intrinsic fluorescence by means of a chemical reaction and so produce a more fluorescent product. In this way, oxidation of the thione group produces the S-oxide of thioguanine [1] which is a fluorophore.

There are several ways of oxidizing thioguanine: with hydrogen peroxide in 50% acetic acid [1], potassium permanganate [2] or potassium chromate [3]; the first offers the greatest sensitivity. It consists of the oxidation of a sample volume v with 0.1 v volumes of hydrogen peroxide in acetate buffer at 50°C for 5 min. After oxidation the solution is made up to 25–50 ml with distilled water. The pH is then adjusted to between 6.0 and 7.0 with

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sodium hydroxide solution, and the fluorescence is measured at 330/415 nm (excitation/emission). However, as the oxidation is performed under highly acidic conditions, it is necessary to neutralize it with concentrated alkali (for instance, 10 M sodium hydroxide), otherwise the sample concentration is diluted. Furthermore, liposomes must be broken up so that the drug is released. Classical methods for disrupting the bilayer structure (action of detergents or organic solvents) are not suitable because they interfere with the final determination.

The study presented here was undertaken to systematically modify the method of the thioguanine oxidation using the method of central composite design, to optimize the assay of thioguanine in a lipid medium after separation of the drug from the lipid.

Experimental

Chemicals

6-Thioguanine (lot 269521) was purchased from Fluka (Germany). In the preparation of liposomes, Lipoid S-100 (Lipoid KG, Ludwigshafen, Germany), a mixture of lipids, whose main component is phosphatidylcholine, was used. C18 Sep-Pak cartridges (Waters–Millipore, USA) were used for the liquid–solid extraction. Methanol and hydrogen peroxide were obtained from Merck (Germany). Water was double-distilled (the second stage being in presence of potassium permanganate), in a borosilicate apparatus, and then purified through a Milli-Q system (Millipore, USA). Acetate buffer, pH 4.7, 310 ± 15 mOSm kg^{-1} was obtained by adding 0.40 g of sodium acetate and 8.47 g of NaCl to 0.30 ml glacial acetic acid and making up to 1 with distilled water. In order to maintain the isotonicity, when the acetate buffer was employed to solubilize the drug, the amount of NaCl added was reduced to 5.67 g. Phosphate buffered solution (PBS), pH 7.2, 310 ± 10 mOsm kg^{-1}, was obtained by dissolving 1.5 g disodium hydrogen phosphate, 0.20 g potassium dihydrogen phosphate, 8 g NaCl and 0.20 g KCl in distilled water, and diluting to 1 l.

Liposome production

Liposomes were obtained by means of the dehydration–rehydration procedure [4]. Briefly, 40 μmole of lipids were dissolved in chloroform. The solution was dried under reduced pressure and then dispersed in 4 ml of acetate buffer containing thioguanine at several concentrations (1, 0.5 and 0.1 mM). After sonication and freeze-drying, small liposomes and unbound drug were separated from thioguanine bound to liposomes by means of ultracentrifugation.

Breakdown of liposomes

After solvating a cartridge of C18 Sep-Pak with 6 ml methanol and flushing it with 6 ml methanol–sodium acetate (pH 4.7; 310 mOsm kg^{-1}) (2:1, v/v), a sample of purified liposomal suspension was loaded onto the cartridge. When the sample had penetrated into the bonded phase, elution with a volume of methanol–sodium acetate led to the breakdown of the vesicles and release of the drug.

Thioguanine determination

Thioguanine eluted from the C18 Sep-Pak cartridge was oxidized with hydrogen peroxide in acetate buffer. The ratio of thioguanine solution to hydrogen peroxide was 10:1, as in the original method [1]. By contrast, the oxidation took 30 min. After this time, three volumes of PBS were added to oxidized thioguanine to give a pH value between 6.0 and 7.0. Fluorescence was measured at 330/415 nm in a Hitachi fluorescence spectrophotometer with 10 nm bandpass, fitted with a 150-W xenon arc lamp. All determinations were carried out at 400 or 700 V lamp voltage.

Optimization of the drug extraction process

Once the thioguanine determination stage was reached, it was decided to check if the extraction capacity of cartridges was influenced by the ratio between the sample loaded and the eluted volume. A two-factorial design at five levels [5] was performed in order to estimate the possibility of interactions between both factors.

Figure 2 shows the two-factor central composite design employed. It consists of a two-level full factorial design (triangles) superimposed on a star design (dots). The centres of the two designs coincide.

Results and Discussion

Fluorescence characteristics

Excitation and emission spectra of thioguanine S-oxide did not show any change by

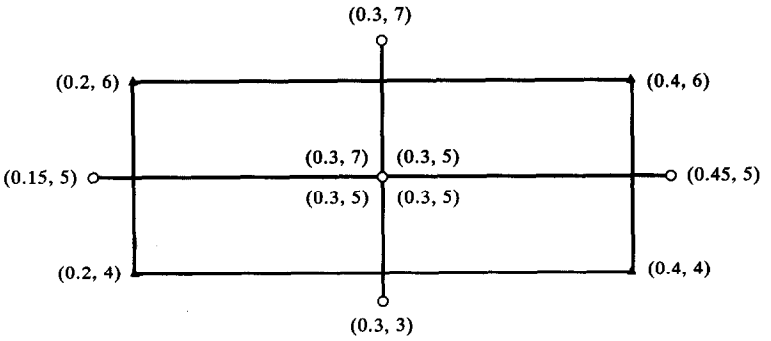

Figure 2
The two-factor central composite design utilized. At every point, the first value states the volume of sample introduced onto the cartridge; the second refers to the volume recovered.

Table 1
Average fluorescence intensity (x), standard deviation (s) and relative standard deviation (RSD) obtained at several times of oxidation

Time (min)	x	s	RSD (%)
15	434	37	8.5
20	567	38	6.7
25	603	45	7.5
30	728	20	2.7
35	731	22	3.0

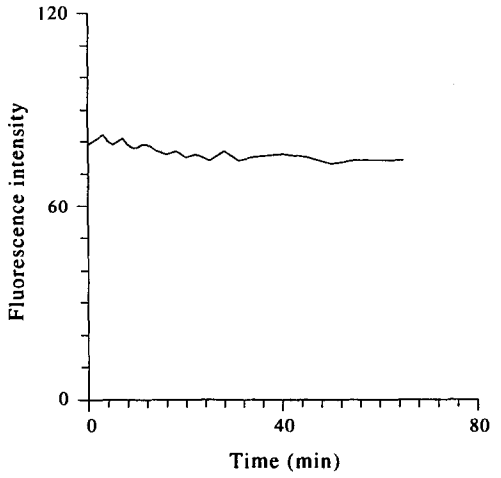

Figure 3
Kinetics of the fluorescence intensity at 415 nm (λ_{ex} = 330 nm).

comparison with the spectra described elsewhere [1]. On checking the oxidation time necessary to achieve stable fluorescence (15, 20, 25, 30 and 35 min), it was shown that 30 min is enough for a complete oxidation (Table 1). After this time, the fluorescence intensity is very stable, as can be seen in Fig. 3, which shows the kinetics of fluorescence measurement performed for more than 1 h.

Validation of the analytical method

Linearity. The linearity of the method was checked by examining the fluorescence intensity over two concentration ranges of thioguanine: from 1 to 60 μM (n = 6) at 400 V; and from 0.01 to 1 μM (n = 6) at 700 V. Each determination was obtained in triplicate. Over both concentration ranges the best-fit line was determined by regression analysis. Both calibration curves afforded a regression coefficient higher than 0.99. A linearity test for the calibration curve was performed by analysis of variance. The ANOVA table for the low-range concentration is given in Table 2. The lower F-value obtained ($3.15 < F(4, 12, 0.05) = 3.29$), points out that the linear model chosen is adequate to describe the relationship between fluorescence intensity and thioguanine concentration.

Table 2
Linearity test of the calibration curve by ANOVA (SS = sum of squares; MS = mean square)

Source of variation	SS	Degrees of freedom	MS	F
Due to regression	SS_{REG} = 30409.47	1	30409.47	2165.00
Variation of group means about the line = lack of fit	SS_{LOF} = 115.01	4	28.74	3.15
Within groups = pure error	SS_{PE} = 109.72	12	9.14	—
Total	SS_T = 30634.0	17	—	—

Precision. The intra-day and between-day precision of the assay were assessed by performing replicate analysis of a standard solution containing thioguanine at 10 μM concentration (1.67 ppm). The RSD values obtained were 1.8% (n = 20) and 2.1% (n = 6), respectively. According to the Horwitz criterion [6], the between-laboratory RSD data at this concentration was 14.8%, while the within-laboratory ranged from 7.4 to 9.9%. Thus, the method was shown to offer adequate precision.

Detection limits and limits of quantitation. The detection limit evaluated for a signal-to-noise ratio of 3 was 0.5 μM. The limit of quantitation was estimated to be 0.08 μM.

Analysis of thioguanine in liposomes

Liquid–solid extraction is an excellent tool to break up the liposomes. This process renders a clear lipid-free solution with a suitable pH value (4.7) for performing the thioguanine oxidation. In this way, it is not necessary to acidify the sample with acetic acid as in the original method. Addition of three volumes of PBS to each volume of oxidized solution is enough to adjust the pH to 6.0–7.0. The final solution results in a stable fluorescence with no significant variation between replicates.

The assay of thioguanine in liposomes has been made with non-purified thioguanine-containing liposomes in order to know exactly the amount of drug present in the sample. After extraction, oxidation and determination, values of fluorescence intensity were interpolated to give the concentration. At any concentration in the range, thioguanine recovery was higher than 85%. The difference between theoretical and experimental values can be explained by the amphiphylic characteristics of the molecule, which implies a relatively high affinity of the drug for an organic medium at pH 4.7 (the partition coefficient of the drug shows a maximum at this pH).

The extraction capacity of the cartridges seems not to be affected by the ratio between the sample loaded and the eluted volume. Analysis of variance of the results obtained showed that there was no interaction between the sample volume loaded and the eluted volume. The polynomial model for describing such a multifactorial chemical system is limited to the following expression: $y = 18.750 + 50.529x_1 - 3.608x_2$, where y is the fluorescence, and x_1 and x_2 are the loaded and the eluted volumes, respectively. Figure 4 shows the response surface for this equation. The ascending plane confirms the lack of interaction.

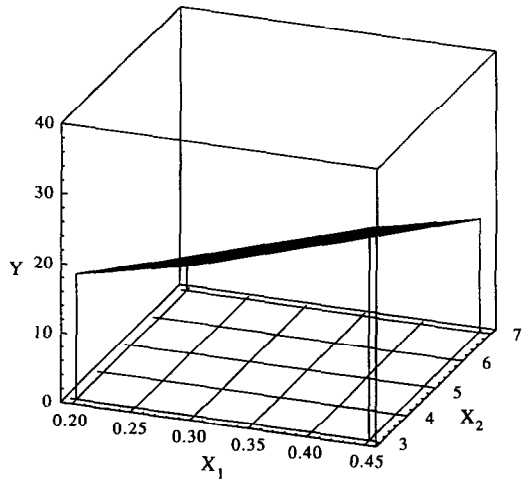

Figure 4
Plot of the system response (fluorescence intensity) vs each of the variables that have an influence on the response (x_1, applied volume and x_2 eluted volume).

Discussion

This paper describes a fluorimetric assay for the measurement of thioguanine in liposomes, employing solid-phase extraction to eliminate the lipid. The method is based on the oxidation of thioguanine by the action of hydrogen peroxide at pH 4.7. The mild conditions of oxidation favour fluorophore stability resulting in stable and concordant fluorescence measurements.

On the other hand, as in any liposomal drug, it is necessary to break down the lipid vesicles as part of the work-up procedure. Triton X-100, the most useful detergent for this purpose, presents a strong, intrinsic fluorescence in the region where thioguanine fluoresces. Organic solvents can disrupt the bilayer membrane but, in this case, the volume relationship organic solvent-to-sample is too large, involving an excessive dilution of the sample as well as a change to a medium unsuitable for performing the oxidation. To solve this problem, liposomes were introduced onto a Waters Sep-Pak C18 minicolumn and after eluting with pH 4.7 acetate buffer, a lipid-free clear solution was obtained. Oxidation can then be made directly. Lipids remaining on the column

can be recovered if chloroform is flushed through the cartridge, if required.

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