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Short Communication

Determination of spirogermanium (2-aza-8-germanspiro[4.5]-decane-2-propanamine-8,8-diethyl-*N,N*-dimethyl dichloride) by fluorometric ion-pair extraction. Application to the uniformity of content of solutions for intravenous injection

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Introduction

Spirogermanium (Fig 1, 2-aza-8-germanspiro[4 5]-decane-2-propanamine-8,8-diethyl-*N,N*-di-methyl dichloride) is a novel cytotoxic agent that contains germanium within a heterocyclic ring structure Although the mechanism of action is uncertain, spirogermanium has shown activity against a number of tumors both *in vitro* [1–7] and *in vivo* [2–4] In addition, several workers have demonstrated the antiarthritic [8, 11] and immunoregulatory [8, 10–12] properties of spirogermanium, while others have shown its activity against *Plasmodium falciparum* [3, 8, 9] Perhaps the most intriguing property of spirogermanium is its lack of bone marrow toxicity [2–4, 13–20] Various pre-clinical investigations [1, 2] predicted that the dose limiting toxicity would be related to its effects on the central nervous system (CNS) This was confirmed subsequently in Phases I and II clinical studies [13–20] which also revealed that the effects of spirogermanium on the CNS are dose dependent, reversible and can be reduced by the use of continuous intravenous infusions

Following the reports of activity against a variety of tumors in Phase I clinical trials [14–16], spirogermanium has entered Phase II studies [17–20], both as a single agent and in combination with other drugs such as 5-fluorouracil [6] The maximum tolerated dose is 80–120 mg m^{-2} given as a 1 h intravenous infusion three times a week [14–20] Despite the number of pre-clinical and clinical studies that have been reported, there is very little information in the literature dealing with the pharmaceutical, biopharma-

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ceutical and pharmacokinetic properties of spirogermanium This is mainly due to the lack of analytical methodology for spirogermanium in formulations, biological fluids and other media of pharmaceutical relevance A complex method for the determination of spirogermanium in plasma, using gas–liquid chromatography has been reported [3], however, work in these laboratories has shown that this method [3] is unsuitable for the routine determination of spirogermanium in aqueous formulations The purpose of the present study was to develop a simple spectrophotometric method for spirogermanium which could be used to determine the uniformity of content of extemporaneously prepared intravenous formulations This methodology was needed since the drug is being used in combination with other drugs, in particular 5-fluorouracil [6] It would be convenient to administer the combinations as single infusions and it is clear [21] that this should not be attempted without first determining the compatibility of spirogermanium with other cytotoxic agents

Experimental

Apparatus

Ultraviolet/visible spectra were obtained using a 1 cm quartz cuvette and an HP 8451A diode array spectrophotometer (Hewlett–Packard, Palo Alto, CA, USA) from 280 to 500 nm Emission spectra were obtained using the same cuvette and a Perkin–Elmer 650-40 fluorescence spectrophotometer from 420 to 600 nm The excitation wavelength was 384 nm with a band width of 10 nm

Chemicals and reagents

All the solvents were HPLC grade and were obtained from Fisher Scientific (St Louis, MO, USA) The acetonitrile was used as received The chloroform was shaken with one half of its volume of water saturated with sodium chloride, dried over calcium chloride and stored in the dark, prior to use The water was doubly-distilled in glass, prior to use The naphthalene-2-sulphonic acid, sodium salt (NS, Fig 1) was obtained from Aldrich Chemical Co (Milwaukee, WI, USA) and recrystallized twice from water The 9,10-dimethoxyanthracene-2-sulphonic acid, sodium salt (DAS, Fig 1) (Fluka Chemical Co , Ronkonkoma, NY, USA), potassium acetate (Aldrich, ACS grade), glacial acetic acid (Fisher Scientific, ACS grade) and tetrabutylammonium hydrogen sulphate (Sigma Chemical Co , St Louis, MO, USA) were used as received Both the spirogermanium dihydrochloride (SG) and the constituted vials of 10 mg ml^{-1} spirogermanium (di)-hydrochloride in 0 9% sodium chloride (Spiro-32®) were kindly provided by Unimed Inc (Somerville, NJ, USA)

Procedures

Spirogermanium or tetrabutylammonium (TBA) was extracted from an acetate buffer (pH 4 0, 0 25 M) into chloroform as ion-pairs with either NS or DAS Following extraction of the ion-pair and separation of the two liquid phases, the absorbance or fluorescence intensity of the chloroform solution was measured The concentrations of NS or DAS in the aqueous buffer were always at least 12 5 times greater than the concentration of the spirogermanium and the TBA For the actual analytical determinations of spirogermanium, the excess of DAS was more than 50-fold For the studies involving absorbance measurements, the final concentrations of NS and DAS in the aqueous phase were 10 and 2 5 mM, respectively Only ion-pairs with DAS were studied

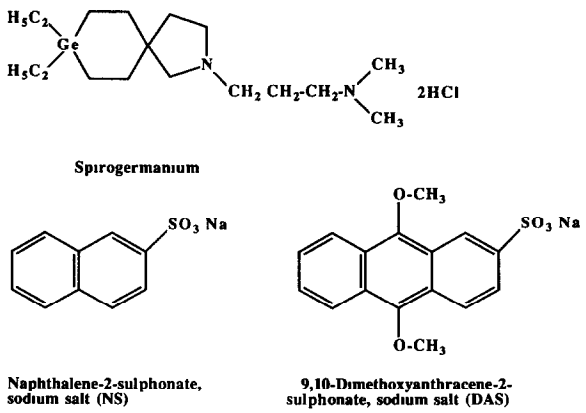

Figure 1
The structures of SG, NS and DAS

by fluorescence, in which case the concentration of DAS in the aqueous phase was 3×10^{-4} M The final extraction procedure for the construction of daily calibration curves and the processing of samples for fluorometric analysis is described below

Calibration curves and sample preparation

Daily calibration curves (n = 5) were prepared from stock solutions containing 5–20 μg ml^{-1} (12–48 μM) spirogermanium dihydrochloride as follows Acetate buffer, 500 μl (0 5 M, pH 4 0), 300 μl of water, 1 ml of 6×10^{-4} M DAS in water and 200 μl of spirogermanium hydrochloride (5–20 μg ml^{-1}, 12–48 μM) were combined in a 12 × 75 mm round bottom polypropylene culture tube (5 ml, polyethylene caps) Dried and alcohol-free chloroform (2 ml) was then added and mixed with the aid of a vortex stirrer for 60 s The two phases were then separated by centrifugation at about 1000 ***g*** for 5 min The aqueous phase was discarded and the fluorescence intensity measured (λ_{ex} = 384 nm, λ_{em} = 450 nm)

Solutions containing spirogermanium which were destined for analysis by absorbance were handled in the same way, except that the concentration of DAS stock solution was 5 mM Samples of spirogermanium (di)hydrochloride for injection were prepared in an identical manner after diluting, in duplicate, with water so that their concentrations fell within the range of the calibration curve The final dilutions of the samples and the calibration solutions were each extracted and measured in triplicate

Results and Discussion

Spirogermanium is an investigational anticancer agent with demonstrated *in vitro* [1–7] and *in vivo* [2–4] activity Unfortunately, its dose limiting toxicity in the CNS has prevented the realization of its full potential in the clinic and it is now being investigated as an adjuvant to other cytotoxic agents The potential usefulness of spirogermanium with other drugs, such as 5-fluorouracil, whose dose limiting toxicity is bone marrow toxicity seems particularly attractive since spirogermanium lacks this particular property [2–4, 13–20] At present, spirogermanium cannot be administered with other chemotherapeutic agents since there is no information available concerning physical and

chemical compatibility This lack of pharmaceutical information is a direct result of the lack of suitable analytical methodology for the determination of spirogermanium in intravenous injections Previous studies in this laboratory aimed at the development of a method based on high-performance liquid chromatography using either electrochemical detection or pre-column derivatization [22] were unsuccessful The present work is concerned with an alternative approach for the determination of spirogermanium which involves the extraction of spirogermanium from aqueous solutions into an organic solvent as an ion-pair with a chromophoric counter ion such as NS or DAS [23] (Fig 1)

Assay development

Spirogermanium is a diacidic base whose pK_a values have not been reported However, based on the pK_as of analogous compounds [24], the values for spirogermanium are likely to be around 8 5 and 10 5 The first pK_a was confirmed by potentiometric titration to be 8 50, however, precipitation of the free base at higher pH values precluded measurement of the second pK_a Studies are ongoing to determine the second pK_a by solubility, using the assay methodology described here Based on these ionization constants, it is reasonable to assume that spirogermanium is fully protonated at pH 4 0 which was chosen for ion-pair extraction experiments The assay procedure was then developed by investigating the effects of organic solvent and the nature of counter ion (NS and DAS) In these experiments, the concentration of the counter ion was maintained well in excess of spirogermanium to ensure complete extraction of the ion-pairs into the organic phase

Initially, experiments were conducted with TBA, a model compound which is known to form extractable ion-pairs with NS and DAS [24] Chloroform and methylene chloride were compared as extraction solvents and chloroform was found to be superior since it showed less of a tendency to form an emulsion Further investigations indicated that DAS was preferred to NS since it has an absorption maximum at 384 nm (Figs 2 and 3) compared with an absorption maximum of 276 nm for NS which was close to the cut-off of halogenated hydrocarbons (244 nm) In addition, NS was found to be hygroscopic and difficult to handle and purify DAS did not show these undesirable properties and had the additional advantage of being fluorescent

Figure 2 shows an absorption spectrum of the ion-pair of TBA DAS extracted from an acetate buffer (pH 4 0, 0 2 M containing 2 5 mM DAS) into an equal volume of chloroform Figure 2 also shows the relationship between absorbance (A) and the initial concentration of TBA ([TBA] in μM) in the aqueous phase which satisfies the linear equation

$$A = 0\ 00659\ [\text{TBA}] + 0\ 0066 \qquad (1)$$
$$r = 0\ 997$$

Figure 3 shows an absorption spectrum for an ion-pair of SG DAS which is virtually identical to that for TBA DAS (Fig 2) In addition, the relationship between absorbance and the concentration of spirogermanium ([SG]) in the aqueous phase is shown in Fig 3 and satisfies the linear equation

$$A = 0\ 00671\ [\text{SG}] - 0\ 0081 \qquad (2)$$
$$r = 0\ 999$$

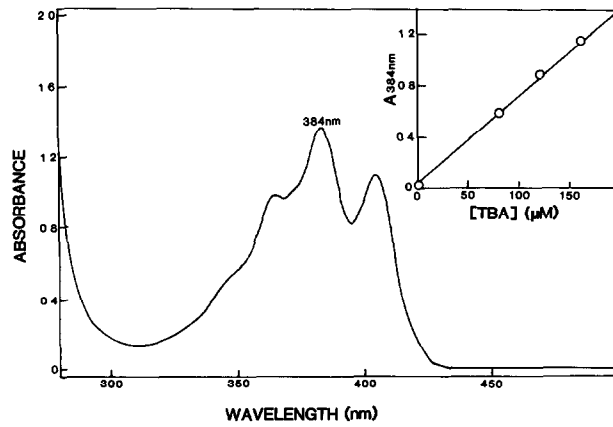

Figure 2
Absorption spectrum of a TBA DAS ion-pair following extraction from an acetate buffer (pH 4 0, 0 25 M) into an equal volume of chloroform The inset graph shows the relationship between the initial concentration of TBA in the aqueous phase and the absorbance of the extracted ion pair Organic phase, chloroform, aqueous phase, 0 25 M acetate buffer (pH 4 0) containing 2 5 mM DAS and various concentrations of tetrabutyl-ammonium hydrogen sulphate

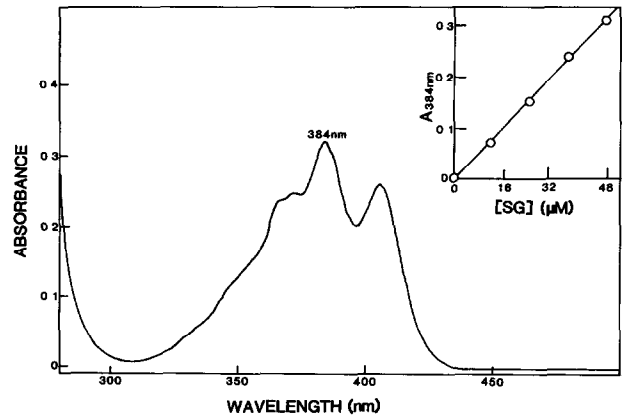

Figure 3
Absorption spectrum of an SG DAS ion-pair following extraction from an acetate buffer (pH 4 0, 0 25 M) into an equal volume of chloroform The inset graph shows the relationship between the initial concentration of SG in the aqueous phase and the absorbance of the extracted ion pair Organic phase, chloroform, aqueous phase, 0 25 M acetate buffer (pH 4 0) containing 2 5 mM DAS and various concentrations of spirogermanium

The similarity of the two slopes strongly suggests that spirogermanium is extracted into a chloroform as a 1 1 association species with DAS and that the charge balance is maintained by a second non-chromophoric anion, probably acetate Further studies to confirm this theory were not conducted

Although a suitable assay procedure based on absorbance measurements was developed, fluorometry was preferred since it was more sensitive and eliminated problems associated with the precipitation of DAS from aqueous solution following extended periods of storage The increase in sensitivity permitted the use of a lower concentration of DAS in the aqueous phase For the processing of samples destined for

analysis by fluorescence, stock solutions of 0 6 mM DAS could be used and stored for extended periods without the formation of precipitate Figure 4 shows an emission spectrum of an SG DAS ion-pair extracted into chloroform and a representative calibration curve which was linear, satisfying the equation

$$F = 103\,[SG] + 0\,000 \qquad (3)$$
$$r = 0\,999,$$

where the concentration of spirogermanium is in $\mu g\ ml^{-1}$

During the assay development, significant problems with adsorption of spirogermanium to glass and certain plastics from chloroform was observed These could be minimized by the use of polyethylene or polypropylene tubes and caps In addition, it was found that the fluorescence intensity of the extracted ion-pair decreased slowly with time if the extract was stored in the fluorometer with the shutter open This phenomenon was attributed to the slow photolytic degradation of the SG DAS ion-pair by the high energy xenon lamp in the fluorometer Consequently, it was important to measure the fluorescence intensity at the same time point, typically 15 s, after opening the shutter

Assay validation and applicability

The assay was validated in terms of linearity, accuracy and precision and its applicability to the determination of intravenous injections containing 10 mg ml^{-1} spirogermanium (di)hydrochloride (Spiro-32®) Figure 4 and equation (3) show that the assay was linear over the range of 0 to 2 $\mu g\ ml^{-1}$ The actual concentrations of drug used for the calibration curve ranged from 0 to 20 $\mu g\ ml^{-1}$ but they experienced a dilution factor of 10 in the aqueous phase, prior to extraction The samples for analysis were diluted with water so that they fell within the linear range of the assay A gradual decline in slope of the calibration curve was experienced, due to the slow decrease in the intensity of the xenon lamp with time The lamp was changed after a decrease of >20% in the response factor (equation 3) was experienced

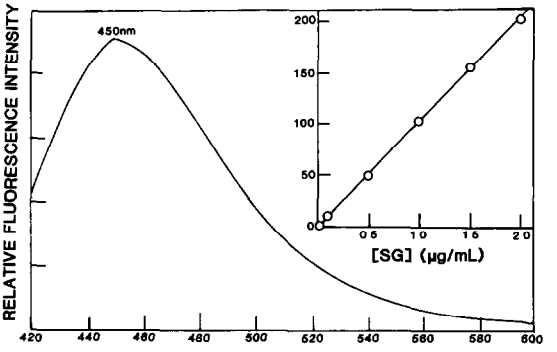

Figure 4
Fluorescence emission spectrum of an SG DAS ion-pair following extraction from an acetate buffer (pH 4 0, 0 25 M) into an equal volume of chloroform The inset graph shows the relationship between the initial concentration of SG in the aqueous phase and the fluorsecence intensity of the extracted ion pair Excitation wavelength 384 nm Organic phase, chloroform, aqueous phase, 0 25 M acetate buffer (pH 4 0) containing 3×10^{-4} mM DAS and various concentrations of spirogermanium

The accuracy of the method was determined to be 97 8% for a spiked placebo This value was obtained by analysing duplicate samples of spirogermanium (di)hydrochloride prepared accurately at a concentration of 10 0 mg ml^{-1} in normal saline (0 9% sodium chloride) The precision of the method was determined by assaying a single vial of Spiro-32® (10 mg ml^{-1}) a total of six times The coefficient of variation of the six determinations was 2 96%, which corresponds to a 95% confidence interval ($\pm stn^{-0\,5}$) of ±0 30 mg ml^{-1} on determinations of solutions containing 10 0 mg ml^{-1} Finally, the uniformity of content of Spiro-32® (10 mg ml^{-1}) was determined by assaying duplicate samples taken from five different vials The average concentration of the vials was found to be 9 81 mg ml^{-1} with a 95% confidence interval of ±0 49 mg ml^{-1}

Conclusions

A simple fluorometric assay for the determination of spirogermanium in aqueous formulations has been developed The procedure is accurate, precise and linear over a concentration range of 0 5 to 2 μg ml^{-1} While not necessarily stability indicating with respect to chemical degradation, the method should prove useful for analytical measurements in which chemical degradation is not a concern The present study has demonstrated the applicability of the method to the testing of uniformity of content It should prove useful in the quality control of extemporaneously prepared intravenous infusions and for testing for physical incompatibilities such as changes in solubility and adsorption to plastic containers Presently, the assay is being used to determine the compatibility of spirogermanium and 5-fluorouracil in small volume parenterals The results of these investigations will be presented elsewhere

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