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Determination of the Antitumor Agent SOAz (1,3,3,5,5 Pentakis(Aziridino)-1λ^6,2,4,6,3 λ^5,5λ^5 Thia-Triazadiphosphorine-1-Oxide) by a Gas Chromatographic Assay Suitable for Pharmacokinetic Studies in Man

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Summary. *A sensitive method, based on capillary gas chromatography using a thermionic detector, has been developed for the new antitumor agent pentakis(aziridino)-thiatriazadiphosphorine-oxide, $(NPAz_2)_2NSOAz$ ('SOAz'), in order to obtain pharmacokinetic data from patients receiving this drug IV in clinical trials. A structural analog of SOAz, $(NPAz_2)_2NSOPh$ ('SOPh'), was used as an internal standard.*

The detection limit of SOAz with this method was 0.01 mg/l for serum and 0.04 mg/l for urine.

The coefficient of variation (n = 10) was 6,0% at 1.5 mg/l in serum and 1.6% at 75.0 mg/l in urine.

Analytical recoveries averaged 89.9% from serum and 86.7% from urine. In two patients treated with subtoxic doses of SOAz (55 mg/m²), serum levels were found ranging from 3.0 to 0.16 mg/l at 10 min and 12 h, respectively, after administration.

This assay seems to be useful for determining SOAz in samples from patients receiving subtoxic doses of SOAz.

Introduction

The thiatriazadiphosphorine oxides constitute a new class of compounds characterized by a heterocyclic ring system consisting of sulfur, nitrogen, and phosphorus [6].

Amongst the numerous compounds the aziridino derivatives [7] have been shown to display cytotoxic activity both in in vitro systems (H. B. Lamberts et al. 1983, unpublished work) and in tumor-bearing mice (L1210 and P388 leukemia and B16 melanoma) [2].

The first of this group of compounds to enter clinical studies is 1,3,3,5,5-pentakis(aziridino),1λ^6,2,4,6,3λ^5,5λ^5-thiatriazadiphosphorine-1-oxide, $(NPAz_2)_2NSOAz$ (SOAz) (Fig. 1). A phase I trial is currently in progress at our institute, and further trials using SOAz and other derivatives are planned. To obtain pharmacokinetic information in the course of those studies a sensitive and reliable assay is needed for quantification of SOAz and related substances in body fluids.

Detection of SOAz after separation by high-performance liquid chromatography (HPLC) seems to be problematic as this compound shows neither absorption of UV or visible light nor fluorescence; furthermore, SOAz lacks electrochemical activity. A derivation procedure using γ-(4-nitro-benzyl) pyridine has been carried out in analogy to a method for the estimation of thiotepa [1]; this method proved to be unreliable and insensitive.

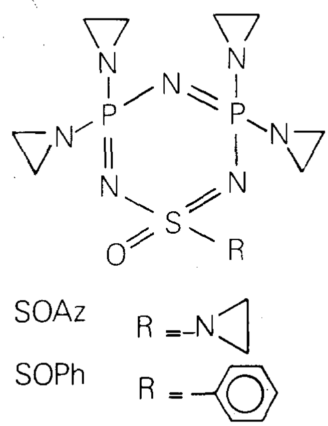

Fig. 1. Structure of SOAz and SOPh

Matsushima and co-workers developed an HPLC method with refraction index detection; by this method they claimed a detection limit for SOAz of 0.5 mg/l. No data concerning reproducibility were reported [5].

In the gas chromatographic assay for SOAz described by Uchida and co-workers [5], separation is carried out by means of a packed column that has to be primed with SOAz prior to each determination. No internal standard is used. The resulting assay is rather insensitive, with a claimed detection limit of 0.5 mg/l.

In our hands this procedure was inadequate because linearity of the calibration curves could not be achieved even at serum levels considerably higher than those typically found in patients treated with subtoxic doses.

Since a more sensitive procedure was required we decided to develop a capillary gas chromatographic assay for the determination of SOAz in body fluids.

Materials and Methods

Reagents. All chemicals used were of reagent grade. Sodium hydrogen carbonate, sodium hydroxide, 1,2-dichloroethane,

Reprint requests should be addressed to S. Rodenhuis and dichloromethane were purchased from Merck, Darmstadt, FRG, and were used without further treatment.

SOAz was supplied by Otsuka Chemical Co., Tokushima, Japan.

SOPh[$(NPAz_2)_2$NSOPh] (Fig. 1) was synthesized at the Department of Inorganic Chemistry, State University of Groningen, The Netherlands.

Newborn bovine serum was purchased from the Flow Laboratories, Irvine, United Kingdom.

Apparatus. A Model 5730A gas chromatograph was used in combination with a Model 18740B capillary column control module and a Model 7671A automatic sampler.

After detection by a Model 18789A N-P-FID, automatic registration and integration of the chromatograms was performed by a laboratory automatic system (32 K 21 MX E Computer) (all from Hewlett Packard Co., Avondale, PA, USA).

The traces were plotted by a standard dual-line compact recorder, with input 10 and 100 mV (PM 8252, Philips N.V., Eindhoven, The Netherlands).

A wall-coated open tubular column, fused silica, 3.3 m × 0.225 mm (inside diameter), liquid phase CP Sil 5, film thickness 0.12 μm with a height equivalent to a theoretical plate of 0.23 mm, was used (Chrompack B.V., Middelburg, The Netherlands).

The split injection method was used. When the column flow of carrier gas (He) was 1 ml/min the column pressure was 0.66 kg/cm^2. A split ratio of 50 : 1 was maintained. The septum vent was 3 ml/min, the bypass flow 30 ml/min, the detector air flow 50 ml/min, and the hydrogen flow 3.1 ml/min. The column temperature was fixed at 170° C, while the injection port and detector temperature were both kept at 250° C.

For identification of the gas chromatographic peaks we used a gas chromatograph-mass spectrometer combination consisting of a Model 3700 gas chromatograph and a MAT 212 mass spectrometer (Finnigan Mat, Bremen, FRG).

The column was identical to the one used for the capillary gas chromatographic assay; the column length was 25 m. The splitless method was used. The initial oven temperature was 100° C, maintained during 3 min and was programmed to rise 20° C/min to a final temperature of 250° C maintained over 6 min. The temperature of the injection port was 270° C, that of the interface 270° C, and that of the separator 350° C (direct conjunction). The carrier gas was He, the column pressure 1.0 kg/cm^2. The source temperature was 300° C, the emission 6 A, and the ion accelerating voltage 3 kV.

Patients. All patients were part of a phase I clinical trial approved by the local ethical committee, and informed consent was obtained from every patient.

Patient A, a 66-year-old male, suffered from disseminated large cell bronchial carcinoma. He had not received any previous chemotherapy. Patient B, a 43-year-old male, had skeletal metastases from a nasopharyngeal carcinoma; previous chemotherapy included *cis*-DDP, bleomycin, and vindesine. Both had normal renal and hepatic function as measured by standard biochemical tests. After rapid IV infusion of SOAz 55 mg/m^2 dissolved in 100 ml 0.9% sodium chloride solution (patient A: 95 mg, and patient B: 100 mg), blood samples were taken 10 min, 2 h, and 12 h after the end of the infusion. From patient A urine was collected from 0 to 180 min and from 3 to 24 h.

Patient C, a 63-year-old male suffering from an adenocarcinoma of the lung, received SOAz 55 mg/m^2 dissolved in 100 ml 0.9% sodium chloride solution (105 mg) by IV infusion over 30 min. Three hours after discontinuation of the infusion, blood and pleural fluid samples were taken simultaneously.

Patient D, a 54-year-old female, had ascites due to hepatic metastases of breast carcinoma. She was treated by rapid IV infusion of SOAz 75 mg/m^2 dissolved in 100 ml 0.9% sodium chloride solution (145 mg); 3.5 h later abdominal paracentesis was performed and blood was taken at the same time.

Treatment of Specimens. Serum, ascites and pleural fluid were stored at −20° C until analysis. Urine samples were stored at 4° C after alkalinization to at least pH 7.5 with sodium hydrogen carbonate.

Assay

Procedure for Determining SOAz in Serum, Ascites and Pleural Fluid: To 0.5 ml serum, ascites or pleural fluid were added 25 μl sodium hydroxide 4 *M* and 100 μl internal standard solution containing SOPh in demineralized water (40 mg/l).

The mixture was vortexed with 5 ml dichloromethane for 30 s and centrifuged at 3,000 rpm for 5 min. The water layer was discarded and the organic layer was decanted into another test tube and evaporated under reduced pressure at 25° C (Vortex Evaporator, Buchler Instruments Inc., Fort Lee, NJ 07024, USA). The residue was dissolved in 200 μl 1,2-dichloroethane. Of this solution, 1 μl was injected into the gas chromatograph.

Procedure for Determining SOAz in Urine: An aliquot of 3 ml urine was centrifuged at 3,000 rpm for 5 min. To 0.1 ml of the clear supernatant was added 0.4 ml demineralized water, and this solution was treated exactly the same as the 0.5-ml serum samples.

Calibration. To calibrate the assay of SOAz in serum, sufficient blank bovine serum was pipetted to 0, 10, 30, 50, 100, 300, and 500 μl of spiked bovine serum (15.0 mg SOAz/l) to give 500 μl; following this procedure these samples contained 0, 0.3, 0.9, 1.5, 3.0, 9.0, and 15.0 mg SOAz/l of serum. To calibrate the assay of SOAz in urine, sufficient blank urine, alkalinized with sodium hydrogen carbonate (pH 7.5) was pipetted to 0, 10, 30, 50, and 100 μl alkalinized spiked urine (pH 7.5, 75 mg/l) and to 10, 30, and 50 μl alkalinized spiked urine (pH 7.5, 750 mg SOAz/l) to give 100 μl; following this procedure the samples contained 0,7.5, 22.5, 37.5, 75, 225, and 375 mg SOAz/l of urine. The foregoing procedures for serum and urine were followed and the calibration curves were plotted, the volumes of spiked serum or urine added being related to the concentrations measured.

The calibration curve slopes and correlation coefficients were calculated by a least-squares procedure.

Recovery Studies. Analytical recoveries for different concentrations of SOAz and of the internal standard SOPh were determined by adding the internal standard of SOAz after the extraction and before the evaporation step and assaying. The relative peak height ratios were calculated (R_1) and compared with the ratio obtained by injection of a mixture of a known amount of SOAz and internal standard onto the column (R_2). The recovery is the ratio between R_1 and R_2 (× 100%).

Results and Discussion

The determination method for SOAz reported below yields highly reproducible results. The coefficient of variation for the serum assay ranges from 3.6% to 8.4% for serum levels of 0.15 to 15.0 mg/l; for the urine assay the coefficients of variation are less than 6.0% for concentrations between 7.5 and 375.0 mg/l; $n = 10$ at each of the concentrations investigated. (Table 1).

The calibration curve for the serum assay is linear, at least in the range 0–15 mg/l; the correlation coefficient is 0.999 and the least-squares regression equation is $y = 0.02869x + 0.0114$. The urine calibration curve shows linearity, at least in the range 0–750 mg/l; the correlation coefficient is 0.9998 and the regression equation is $y = 0.69583x - 1.7823$.

The detection limits of SOAz are 0.01 mg/l and 0.04 mg/l for serum and urine, respectively; since these values were obtained at a gas chromatographic attenuation of 8×1, even lower concentrations of SOAz may be detectable if attenuation is reduced.

Analytical recovery of SOAz from serum averaged 89.9% and that from urine, 86.7%; recovery of the internal standard SOPh from serum and urine was 77.2% and 75.4%, respectively (Table 2). The somewhat wide variability of recovery of SOAz at different concentrations can be ascribed to unavoidable inaccuracies in the determination of recovery. In establishment of the calibration curve, however, the internal standard is immediately added to the sample and is subject to the same fluctuations of extraction as SOAz. Therefore, the accuracy of calibration is far less affected than one would expect on the basis of the recovery studies alone.

Only a short single extraction and evaporation step is required before chromatography. Due to the use of a short capillary column (3 m) the retention time (SOAz, 0.5 min; internal standard SOPh, 1.8 min) are considerably reduced with no need to use very high temperatures (Fig. 2). In addition, time-consuming priming of the column with SOAz is unnecessary. Overnight determination of a large number of samples is feasible, using computer-controlled analysis and integration of the traces. During the development of the method a number of compounds were evaluated for their suitability for use as an internal standard. Several structural analogs of SOAz were tried and one of these (SOPh) was finally selected because of its favorable retention time and extraction coefficient. Since each of these compounds produces one sharp peak on the chromatogram, it is to be expected that this assay will be suitable for quantification of other S,N,P-ring systems with no or minor modifications.

Since SOAz is known to be unstable at temperatures exceeding 130° C [7], the question arose as to whether the traces obtained after separation by a column at 170° C represent SOAz itself or one of its decomposition products. Therefore, mass spectrometric analysis of serum and urine samples was performed after separation by a capillary column at 250° C and the results were compared with mass spectra obtained with unprocessed SOAz: the three spectra were identical and were all characterized by a low-intensity peak of $m/e = 362$ (M^+) and a very pronounced peak corresponding to $m/e = 320$, as a result of the loss of one aziridino group. It is reasonable to assume that due to its low concentration, SOAz remains stable in a capillary column at the temperature used.

Serum levels 10 min, 2 h and 12 h after IV administration of SOAz (55 mg/m^2) in patient A were 2.29, 1.04, and 0.16

Table 1. Coefficients of variation for analysis of different concentrations of SOAz ($n = 10$)

Serum[a]		Urine[b]	
Concentration (mg/l)	CV (%)	Concentration (mg/l)	CV (%)
0.15	8.4	7.5	3.5
0.75	7.4	75.0	1.6
1.50	6.0	375.0	5.6
15.0	3.6		

[a] In fortified calf's serum
[b] In fortified urine, alkalinized by sodium hydrogen carbonate to pH 8.1

Table 2. Recovery of SOAz at different concentrations and of the internal standard SOPh from serum and urine

	Compound	(mg/l)	Recovery (%)	CV (%)	n
Serum	SOAz	1.5	95.1	5.0	10
	SOAz	15.0	84.4	5.2	10
	SOPh[a]		77.2	8.2	12
Urine	SOAz	7.5	79.7	8.0	7
	SOAz	75.0	85.1	3.2	12
	SOAz	375.0	95.4	11.3	12
	SOPh[b]		75.4	6.9	11

[a] In calf's serum to which 100 µl of a solution of SOPh in demineralized water (42.60 mg/l) was added
[b] In urine alkalinized by sodium hydrogen carbonate (pH 8.1) to which 100 µl of a solution of SOPh in demineralized water (42.60 mg/l) was added

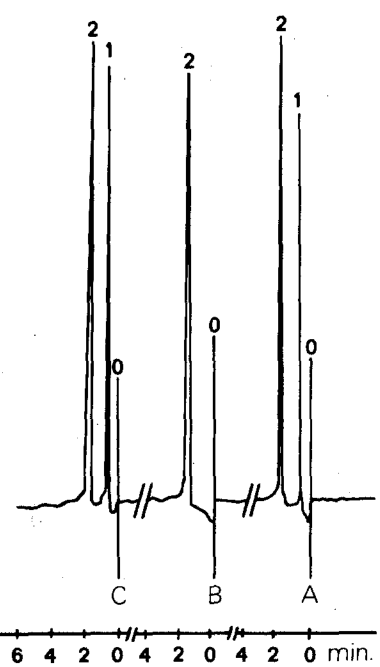

Fig. 2. Chromatograms of serum (A and B) and urine (C) of patient A (gas chromatographic attenuation 32 × 1). 0, Injection; 1, SOAz (A 2.2 mg/l : C 13.9 mg/l); 2, internal standard SOPh

mg/l, respectively. In patient B, who received the same dose, the corresponding serum levels were 3.01, 1.47, and 0.25 mg/l. Patient A excreted 29.47 mg of unchanged drug in his urine in the first 3 h and 20.35 mg in the next 21 h. The concentrations in the urine samples were 185.35 and 14.45 mg/l, respectively.

The pleural fluid of patient C contained 0.79 mg SOAz/l with a simultaneous serum level of 0.65 mg/l. In the ascites of patient D a SOAz concentration of 0.92 mg/l was found, with a simultaneous serum level of 2.80 mg/l.

These preliminary pharmacokinetic data of patients enrolled in a phase I clinical trial indicate that serum levels decrease rapidly during the first 12 h after administration. It is of interest that these patients experienced no or only mild toxicity at these dose levels, indicating that this method has sufficient sensitivity for clinical use [3]. About 50% of the SOAz administered was excreted unaltered in the urine of patient A within the first 24 h. Apparently the drug readily enters pleural fluid and ascites. All concentrations found in these biological fluids fall well within the range of the assay. More details on the clinical pharmacokinetics of SOAz are presented elsewhere in this issue [4].

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