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DETERMINATION OF SPARSOMYCIN IN PLASMA AND URINE OF THE DOG BY MEANS OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FIRST PHARMACOKINETIC RESULTS

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SUMMARY

Sparsomycin — an antibiotic with marked cytostatic activity — was the subject of a clinical Phase I study in 1964. The structure of sparsomycin was elucidated in 1970 and its first total synthesis was reported in 1981. Here we describe a sensitive high-performance liquid chromatographic method of determination. The detection limit was found to be 10 ng/ml of plasma and 20 ng/ml of urine. With this procedure sparsomycin and isosparsomycin can readily be separated.

In addition, we performed a first pharmacokinetic study in the dog and found a half-life time $t_{1/2}$ β of 1.1 h. Only 25% of the administered dose could be recovered in the urine as sparsomycin.

We consider that by now many prerequisites for further preclinical studies have been achieved, and the results of these studies will determine whether sparsomycin deserves reintroduction into clinical use.

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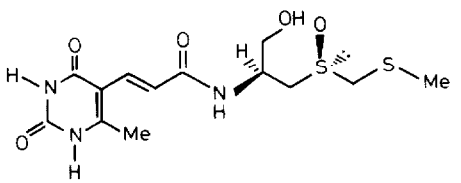
INTRODUCTION

Sparsomycin, an antibiotic, has been known since the early sixties as an in vitro and in vivo protein-synthesis inhibitor [1—5]. It has been found to block peptide-bond formation and thus to prevent the transfer of amino acids from RNA to the nascent polypeptide chains [3]. In addition, sparsomycin has shown cytostatic activity in a number of in vivo tumor systems [1, 6] as well as activity in a cell tissue culture of KB human epidermoid carcinoma [1]. Therefore, it was introduced in 1964 in a clinical Phase I study. Two of the five patients of this study developed an ocular toxicity, probably caused by sparsomycin, and so this Phase I study was stopped prematurely [6, 7].

Until recently, sparsomycin was only available from natural sources; it was isolated as a fermentation product of *Streptomyces sparsogenes* [8]. In 1970 Wiley and MacKeller [9] succeeded in elucidating the structure of sparsomycin and in 1981 two of us [10, 11] reported its synthesis (Fig. 1). In the course of its synthesis it was noticed, that under the influence of UV light the alkene double bond isomerises easily to a *cis* configuration, yielding isosparsomycin. In an in vitro clonogenic cell assay with L 1210 cells, sparsomycin was found to be the cytostatic active compound, whereas isosparsomycin was devoid of activity in this assay [12]. These findings are noteworthy, as sparsomycin from natural sources was found to contain appreciable amounts of isosparsomycin.



Sparsomycin

Fig. 1 Structure of sparsomycin.

Knowledge of the mechanism of action of sparsomycin, which is different from that of the cytostatics in common use, together with detailed knowledge of its structure and synthesis, which allows, for example, the synthesis of sparsomycin analogues, and the possibility to obtain highly purified sparsomycin, justify a preclinical reinvestigation of this antibiotic. Such an approach has also been proposed by the authors of the Phase I study [6]. To be able to undertake pharmacokinetic studies of its distribution, metabolism and excretion we report here a new, sensitive high-performance liquid chromatographic (HPLC) method for the determination of sparsomycin. The detection limit of our method is 10 ng/ml of plasma and 20 ng/ml of urine; and the method was applied in a first pharmacokinetic study in the dog. Until now, sparsomycin could only be detected in the microgram range, as by the HPLC method described by Chan et al. [13].

MATERIALS AND METHODS

Sparsomycin concentrations were determined by a two-column HPLC back-

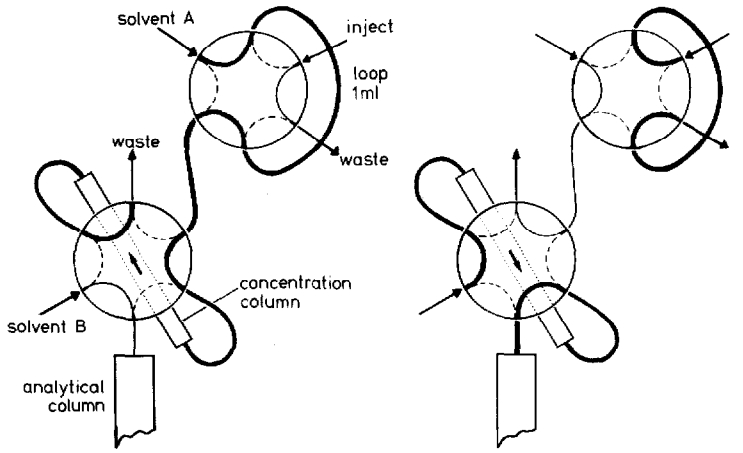


Fig. 2. Left: valves in concentration position. Right: valves in injection position [14].

flush method. By using a concentration column, the samples could be cleaned from interfering peaks and concentrated (Fig. 2) [14].

Equipment

The chromatography equipment consisted of a double head solvent pump for solvent A and solvent B (Orlita, DHP 1515, Bakker & Co., Zwijndrecht, The Netherlands). Two sampling valves (Valco, Houston, TX, U.S.A.) were used as in Fig. 2: left side = concentration position, right side = injection position. The concentration column (5 cm × 3.0 mm I.D.) was filled with LiChrosorb RP-8, 10 μm; the analytical column (15 cm × 4.6 mm I.D.) was packed with reversed-phase material Cp^{tm} Spher C8, spherical particle size 8 μm (both from Chrompack, Middelburg, The Netherlands). A spectrophotometric detector was used at a wavelength of 300 nm (Spectroflow monitor SF 770 + UV monochromator GM 770, Schoeffel, The Netherlands).

Solvents and concentration method

Solvent A, demineralized water, was flushed via the injection loop through the concentration column with a flow-rate of 2 ml/min. Plasma samples were flushed with 9 ml of water, urine samples with 11 ml of water. After 4.5 min and 5.5 min, respectively, the concentration column was flushed back with solvent B, methanol—demineralized water (15:85), onto the analytical column with a flow-rate of 1.3—1.5 ml/min.

Drugs

Sparsomycin was synthesized as described earlier [10]; an aqueous solution, which was protected from light, was used for calibration and for the animal study.

Dog

A beagle dog (No. 686), bred by the central animal laboratory of the University of Nijmegen, was kept under anaesthesia for the first 8 h. A continuous infusion (800 ml of 5% glucose) was administered over this period

of time via a catheter placed in the front leg to achieve a higher urine flow. Sparsomycin (0.7 mg/kg body weight) was injected via this catheter as a bolus injection. Plasma samples were drawn from an indwelling catheter in the jugular vein at 5, 15, 30, 45, and 60 min and subsequently each hour into heparinized tubes, which were immediately centrifuged and frozen (-20°C). For the next two days the dog was kept in a metabolic cage, to obtain further spontaneously voided urine samples.

Sample preparation

All samples were kept protected from light.

Plasma. To 1 ml of plasma, stirred on a vortex mixer, 1 ml of 10% trichloroacetic acid in water was added. After centrifugation at 1500 *g* for 5 min, 1.5 ml of the supernatant (protein-free) were transferred into a glass tube; 1 ml of chloroform was added and the resulting mixture was shaken on a vortex mixer. After a second centrifugation at 1500 *g* for 5 min, 1 ml of the upper (aqueous) layer was injected into the sampling loop.

Urine. Samples were diluted with demineralized water and also "cleaned" with chloroform. The early urine samples could be diluted 1 in 5, the late ones only 1 in 2. Samples of 1.5 ml of these dilutions were washed by mixing with 1 ml of chloroform. After centrifugation at 1500 *g* for 5 min, 1 ml of the upper layer was injected into the sampling loop.

Calibration curves. These were constructed by adding known amounts of sparsomycin to plasma or urine and the resulting solutions were treated as described above.

RESULTS

Detection of sparsomycin and isosparsomycin

UV absorption maxima. Sparsomycin shows absorption maxima at 300 nm and 188 nm. At 188 nm the extinction coefficients of isosparsomycin and

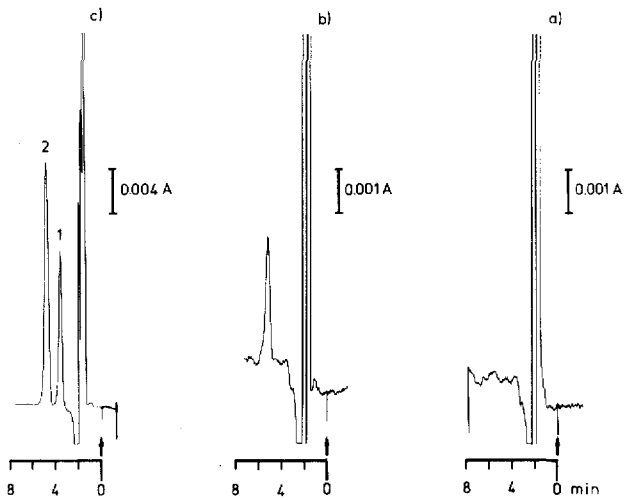


Fig. 3. HPLC of (a) blank plasma, (b) blank plasma spiked with 40 ng/ml sparsomycin, (c) sparsomycin (2) and isosparsomycin (1) in water.

sparsomycin are identical. However, at 300 nm the extinction coefficient of isosparsomycin is about one third that of sparsomycin. Therefore the ratio sparsomycin/isosparsomycin could be determined at 188 nm (see below and Fig. 4).

Chromatogram. Fig. 3 shows HPLC chromatograms detected at a wavelength of 300 nm; the flow rate of solvent B was 1.5 ml/min. In Fig. 3c it can be seen that sparsomycin (peak No. 2) and isosparsomycin (peak No. 1) can easily be separated by the applied procedure. The retention times are 4.8 min and 3.6 min, respectively. Chromatogram b is from a plasma sample spiked with 40 ng/ml sparsomycin. In comparison, chromatogram a is from blank plasma.

Detection limit. The lowest concentrations of sparsomycin that could be determined with the described method were 10 ng/ml of plasma and 20 ng/ml of urine; with urine samples there were more interfering peaks in the chromatogram.

Stability of sparsomycin during the work-up procedure

Towards light. To study the influence of light upon sparsomycin, we exposed an aqueous solution of sparsomycin (4 μg/ml) in a glass container, covered with parafilm, to normal laboratory light conditions. Another sample of the same solution was stored, protected from light, in the refrigerator. The concentrations of sparsomycin and isosparsomycin were determined daily and compared to the reference solution. In Fig. 4 the light-induced isomerisation of sparsomycin (*trans*) into isosparsomycin (*cis*) as a function of time is shown. Under the described conditions 50% of isosparsomycin was formed from sparsomycin in 3.6 days.

Towards thawing and freezing. To determine the stability of sparsomycin in frozen plasma, samples were spiked with 200 ng/ml and kept frozen for two weeks. Samples were then thawed and refrozen one to three times. After each thawing HPLC analysis was done in triplicate and compared with freshly

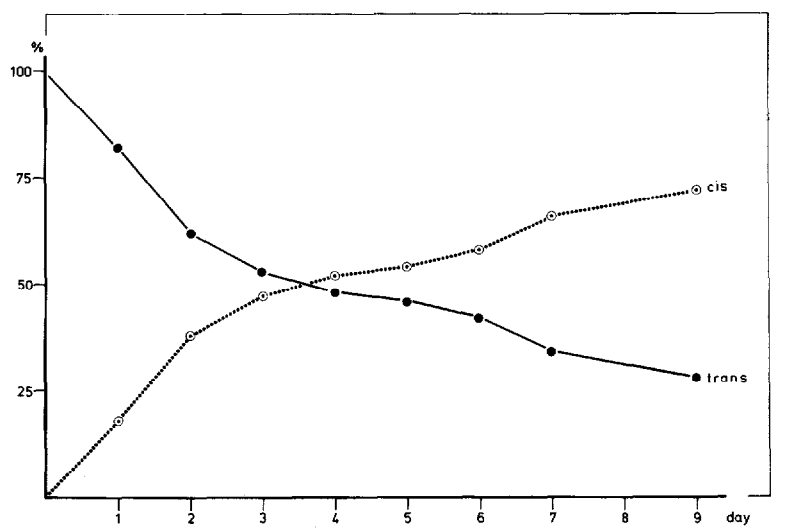


Fig. 4. Light-induced isomerisation of sparsomycin (*trans*) into isosparsomycin (*cis*); 50% isomerisation occurred within 3.6 days.

spiked plasma samples. The amount of sparsomycin determined in the samples that had been kept frozen was 92 ± 5% of the amount of sparsomycin determined in the samples that had been freshly spiked. No relation could be detected between the number of times that samples were frozen and the degradation of sparsomycin.

At room temperature. Samples of an aqueous sparsomycin solution, which had been kept protected from light for three days, showed no decrease in concentration of sparsomycin.

Recovery and reproducibility

The recovery of sparsomycin added to plasma in the concentration range 10—3500 ng/ml was found to be 70 ± 4% S.D.; in urine the recovery was 100 ± 2% S.D. (triplicate analyses). As expected from its polar structure, sparsomycin is not extracted into the chloroform phase during the clean-up procedure for plasma and urine. Repeated injections of samples with the same concentration showed a change in peak hight of ± 2% S.D. in the concentration range 10—3500 ng/ml.

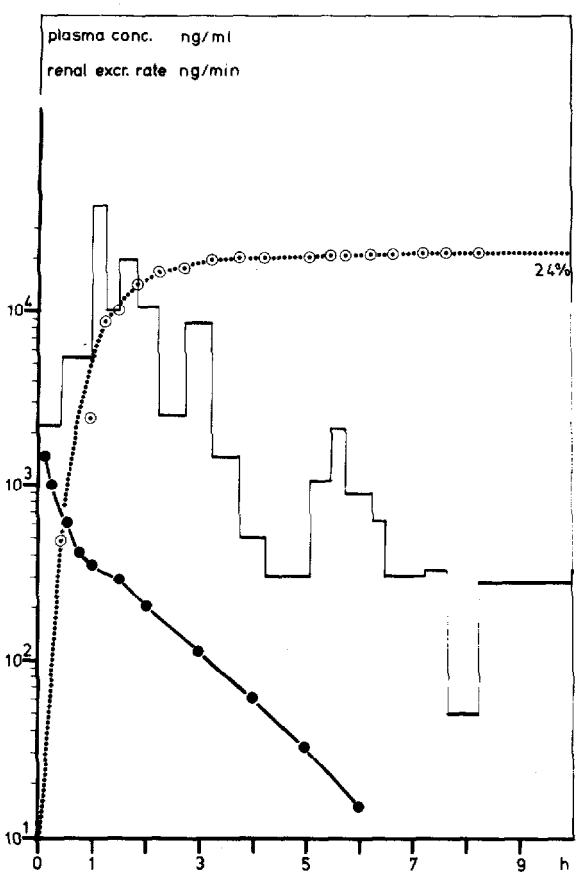


Fig. 5. (●——●), plasma concentration versus time after 0.7 mg/kg sparsomycin as intravenous bolus injection in the dog. (——), renal excretion rate per urine portion. (⊙···⊙), cumulative renal excretion of sparsomycin.

Results of the pharmacokinetic study in the dog

Plasma concentrations. In Fig. 5 the plasma concentrations of sparsomycin versus time are shown after an intravenous bolus injection of 0.7 mg/kg body weight in the dog. Two phases can be discriminated: (1) 5 min to 45 min with a half-life time $t_{1/2}$ α of 0.13 h; (2) 45 min to 6 h with a half-life time $t_{1/2}$ β of 1.1 h.

Renal excretion rate and recovery in urine. In Fig. 5 renal excretion rates per urine portion versus time are shown. The cumulative excretion of sparsomycin (dotted line) accounts for only 24% of the administered dose in the period of 10 h, as shown. (A total of 25% could be recovered after 24 h of urine collection.)

Further pharmacokinetic results. Based on the two-compartment model, the following parameters from the pharmacokinetic profile of the dog have been calculated: volume of distribution of the central compartment = 4.7 l; area under the plasma concentration curve = 1.4 mg h/l; total body clearance = 117 ml/min; renal clearance, based on total plasma concentration = 48 ml/min.

In Fig. 6 the flow dependency of the renal clearance of sparsomycin is shown in the range 0.2—4.9 ml/min urine flow (r = 0.87).

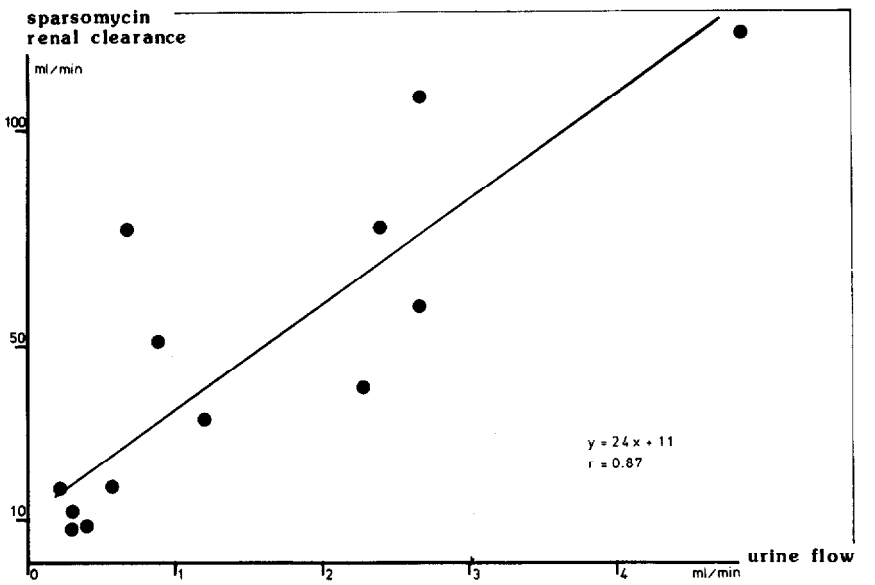


Fig. 6. Renal clearance of sparsomycin in the dog in relation to urine flow.

Clinical observations in the dog

Based on the reported side-effects of sparsomycin in animal studies [6], this pharmacokinetic study in the dog was accompanied by blood chemistry studies, in search of renal, hepatic and bone marrow toxicity. The following changes were observed:

(1) A drop of total serum protein from 65 to 50 g/l on the second day after sparsomycin administration. After one week serum protein was back to 58 g/l.

(2) A marked drop in the thrombocyte count from 300 × 10^9 per l towards 139 × 10^9 per l at the first day after sparsomycin administration and a recovery within a week.

(3) Absence of hepatic and renal toxicity as controlled by means of alkaline phosphatase, serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), blood urea nitrogen (BUN) and serum creatinine.

(4) The dog showed a high degree of tiredness during the first week after the administration of sparsomycin.

DISCUSSION

Method of determination

As we have shown, the described method of determination is sensitive enough to be used in further preclinical pharmacokinetic studies. In addition, this method may also be of value on a preparative scale, to separate sparsomycin from isosparsomycin in the reaction mixture of the synthesis of sparsomycin.

Previously used doses in patients

In Table I we list the doses of sparsomycin that have been used in patients. Two patients, nos. 2 and 3, of the Phase I study developed ocular toxicity after 13 and 15 consecutive days of treatment with sparsomycin (the natural fermentation product). The pharmacokinetics of its distribution, metabolism and excretion after this consecutive way of administration could differ markedly from the usual single-dose administration used in most of the test systems.

TABLE I

PREVIOUSLY USED IN VIVO DOSAGES OF SPARSOMYCIN

Subjects studied	Total dose (mg/kg)	Reference
11 of 20 different tumors in animals significantly inhibited	0.25—1.0	1962 [9]
Pat. 1 10 consecutive days of administration	0.085	1964 [3]
Pat. 2 13 consecutive days of administration	0.24	Ocular toxicity [3, 7]
Pat. 3 15 consecutive days of administration	0.15	Ocular toxicity [3, 7]
Pat. 4 10 consecutive days of administration	0.136	[3]
Pat. 5 17 consecutive days of administration	0.154	[3]
Dog 868 one intravenous bolus	0.7	Present paper

Side-effects from sparsomycin, and its pharmacokinetics in the dog

The retinopathy [6, 7] is of course the most serious side-effect of sparsomycin that needs to be further evaluated. Furthermore, liver function impairment is a likely result of sparsomycin treatment; elevation of SGOT and/or alkaline phosphatase was reported in four of the five patients after treatment with sparsomycin had started [6].

Our first study in the dog was designed to obtain a pharmacokinetic profile of sparsomycin. It was found that sparsomycin has a very short half-life time of 1.1 h; and only 25% of the administered dose could be recovered in the urine as

the parent compound. Consequently, there may be other routes of excretion, and/or metabolites of sparsomycin may be formed which can not yet be detected by our procedure. Although no major emphasis was put on clinical issues in this study, hepatic toxicity was not observed in the dog.

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