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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF A NEW β-LACTAM ANTIBIOTIC, 6059-S (MOXALACTAM)

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SUMMARY

Reversed-phase high-performance liquid chromatography was applied to the quantitative determination of a new β-lactam antibiotic, 6059-S, and its *R*- and *S*-epimers were resolved. The procedure was also applied to pharmaceuticals and human urine samples. Chromatographic separation was effected on a bonded hydrophobic stationary phase with two mobile phases: methanol—phosphate buffer for the resolution of the epimers and methanol—tetra-*n*-butylammonium phosphate for the quantitation of 6059-S. For the determination of 6059-S in human urine, the latter mobile phase was used successfully without interference by the other urine components. An in vivo experiment was conducted by administering intravenously 1 g of 6059-S to seven volunteers and analysing their urine by chromatographic and microbiological assays, and a comparison of the results gave a correlation coefficient of 0.9954. One-compartment model analysis of the time-course data revealed that 6059-S was excreted in urine intact with a rate constant of 0.433 h^{-1}.

INTRODUCTION

A new semi-synthetic β-lactam antibiotic, 7β-[2-carboxy-2-(4-hydroxyphenyl)acetamido]-7α-methoxy-3-{[(1-methyl-1H-tetrazol-5-yl)-thio]-methyl}-1-oxa-1-dethia-3-cephem-4-carboxylic acid disodium salt. 6059-S (Moxalactam) (I) [1], was discovered to be highly active against a broad range of gram-negative microorganisms, including those resistant to other cephalosporins, and to have a wide antibacterial spectrum [2].

Determinations of antibiotic drugs are conventionally performed by microbiological assay. Such methods require a relatively long time and sometimes result in difficulties when more than one antibiotic drug is administered or an antibiotic is accompanied by one or more active decomposition products. Sometimes 6059-S is accompanied by a small amount of a microbiologically

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active decomposition product, decarboxy-6059-S (II), and a non-active one, 1-methyl-1H-tetrazole-5-thiol (III).

Diastereoisomers of many penicillins or cephalosporins occur. As shown in Fig. 1, 6059-S, which closely resembles cephalosporins or cephamycins in structure, also consists of *R*- and *S*-epimers owing to an asymmetric carbon atom in the side-chain. In recent years, much work has been published on the high-performance liquid chromatographic (HPLC) separation of β-lactam antibiotics. However, no HPLC separation of their diastereoisomers has been reported except for ampicillin [3], cephalexin [3], 7-ureidoacetamidocephalosporins [4] and carbenicillin [5].

This paper describes the determination of 6059-S and the separation of its *R*- and *S*-epimers in the quality control of pure substances and in human urine by reversed-phase HPLC. The results obtained by HPLC and microbiological assays are compared.

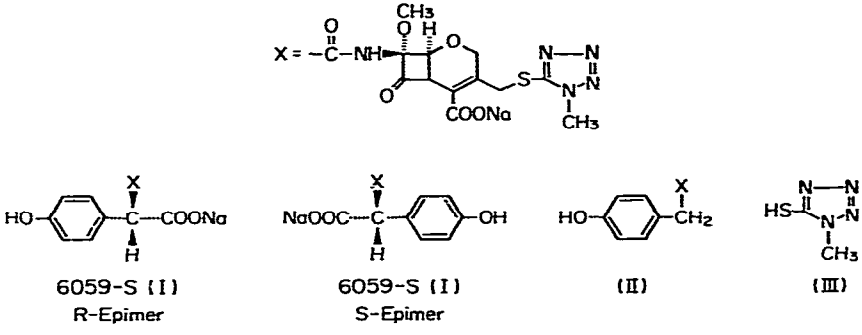

Fig. 1. Structures of 6059-S and related compounds.

EXPERIMENTAL

Materials and reagents

The compounds 6059-S (I), decarboxy-6059-S (II) and 1-methyl-1H-tetrazole-5-thiol (III) were synthesized at Shionogi Research Laboratory (Osaka, Japan). Guaranteed-reagent grade monobasic potassium phosphate (Kanto, Tokyo, Japan), monobasic sodium phosphate (Wako, Osaka, Japan) and dibasic sodium phosphate (Wako) and reagent-grade *o*- and *p*-acetanisidide (Eastman-Kodak, Rochester, NY, U.S.A.) were used. A 10% solution of reagent-grade tetra-*n*-butylammonium hydroxide (Wako) was used as a constituent of the mobile phase in paired-ion chromatography. Water was purified by using an ion-exchange column, reverse osmosis and finally a single distillation. HPLC-quality methanol (Nakarai, Kyoto, Japan) was used.

Apparatus

The liquid chromatograph consisted of a Waters Model 6000A pump, a Type U6K injector (Waters Assoc., Milford, MA, U.S.A.) or a Rheodyne Model 7120 injector, and a Model 440 UV detector (Waters Assoc.) or a Japan Spectrooptics UVIDEC-100 variable-wavelength UV detector. A pre-

packed column of Nucleosil $10C_{18}$, particle size 10 μm (30 cm × 4 mm I.D.), was obtained from Macherey, Nagel & Co. (Düren, G.F.R.). For urine analysis, a guard column packed with Nucleosil $10C_{18}$ in a 5 cm × 4 mm I.D. stainless-steel tube was connected to the analytical column. Quantitation was based on integration of peak areas using a Chromatopac EIA (Shimadzu, Kyoto, Japan).

Chromatographic procedure

Two mobile phases were applied: methanol—0.05 *M* monobasic potassium phosphate solution (5:95) adjusted to pH 6.5 (eluent A) and methanol—0.005 *M* tetra-*n*-butylammonium phosphate solution (25:75) which was prepared by combining a 10% solution of tetra-*n*-butylammonium hydroxide with a mixed solution of dibasic sodium phosphate and monobasic sodium phosphate to make the pH 6.0 (eluent B).

The mobile phase was passed through a membrane filter (0.4 μm) to remove any particulate matter and dissolved gases prior to use. All experiments were run at room temperature. The flow-rates were 2.0 ml/min for eluent A and 1.0 ml/min for eluent B, and the inlet pressures were about 14 and about 10 MPa, respectively. The detector was operated at 254 nm.

Determination of R- and S-epimer ratios and quantitation of 6059-S in pharmaceutical raw materials

Approximately 0.5 mg of sample was dissolved in 1 ml of distilled water at room temperature. Immediately, 5 μl of the sample solution were introduced on to the chromatographic column under the above operating parameters using eluent A. Ratios of the *R*- to the *S*-epimer were determined from the ratios of their peak areas.

For quantitative determination, a 60 mg/l internal standard solution of *p*- or *o*-acetanisidide was prepared. Reference 6059-S, with a known water content, was accurately weighed into a volumetric flask, then dissolved and diluted as required with the internal standard solution to give a range of concentrations from 0.5 to 0.1 mg/ml. A 5-μl volume of a standard solution of 6059-S was injected into the column under the above operating parameters using eluent B. Calibration graphs for 6059-S were prepared by plotting the standard sample concentration corrected for the water content against the ratio of the sum of the peak areas of the *R*- and *S*-epimers to the internal standard peak area. Sample solutions of pharmaceutical raw materials were prepared in a similar manner to those of the standards and subjected to HPLC under the same conditions as the standards.

In vivo experiments and determination in human urine

The in vivo experiments were conducted with two groups of three (T) and four (F) healthy male volunteers. In each experiment, 1 g of 6059-S was given intravenously to each volunteer. The ratios of the *R*-epimer to the *S*-epimer of the dosed drugs were 1.11 and 1.05 for the T and F groups, respectively. In the T group, urine samples were taken just before and at 1, 2, 4, 6, 8 and 12 h after administration, frozen, stored at −20°C and measured within 1 week. On another day, urine samples of the F group were

taken just before and at 1, 2, 4, 6 and 8 h after drug administration, and analysed in the same way as for the T group.

After thawing, 1—2-ml urine samples were passed through Sep-Pak cartridges (Waters Assoc.) for preliminary sample clean-up, and the eluates were injected directly into a liquid chromatograph under the above operating parameters using eluent B.

For the calibration procedure, 1 g from the same batch as the dosage drug was dissolved in distilled water and diluted with water to give a concentration range of 10—0.1 mg/ml. Calibration graphs for 6059-S were prepared for the ranges 10—2, 3—0.3 and 1—0.1 mg/ml by plotting the standard sample concentration against the sum of the peak areas of *R*- and *S*-epimers for the concentration range desired from the predicted concentration in urine samples. Ratios of the *R*- to the *S*-epimer were determined from the ratios of their peak areas together with the quantitation of 6059-S.

Microbiological assay of 6059-S

Concentrations of 6059-S were determined by the band culture assay method, using as the test organism *E. coli* 7437, which has been described previously [2]. Standard solutions of 6059-S were prepared at concentrations of 8, 4, 2, 1, 0.5 and 0.25 μg/ml by dissolving 1 g from the same batch as the dosage drug in a 0.1 *M* potassium phosphate buffer solution (pH 7.0). The urine samples were diluted to a concentration below 8 μg/ml prior to assay.

RESULTS AND DISCUSSION

Separation of R- and S-epimers

There are many diastereoisomers of penicillin, cephalosporin and cephamycin antibiotics owing to the existence of an asymmetric carbon in the side-chain of the β-lactam ring. Determination of the diastereoisomeric purity of antibiotics is important in order to evaluate the antimicrobial activity of the isomers. Recently, Salto [3] separated completely the diastereoisomers of ampicillin and cephalexin by reversed-phase chromatography, and Young [4] also separated some diastereoisomeric 7-ureidoacetamido cephalosporins in a similar manner.

We were able to separate the diastereoisomers of 6059-S completely by reversed-phase chromatography using 0.05 *M* phosphate buffer (pH 6.5) containing 5% methanol, as shown in Fig. 2. Under these conditions, an impurity, 1-methyl-1H-tetrazole-5-thiol (III), was eluted early but another impurity, decarboxy-6059-S (II), was not eluted, probably owing to the difference in the degree of hydration based on monobasic and dibasic acids. With gradient chromatography, increasing the methanol content in the same mobile phase, the peak of decarboxy-6059-S (II) is, of course, observed.

The former of the two peaks of 6059-S on the chromatogram shown in Fig. 2 was identified as that of the *R*-epimer of 6059-S, based on comparison with the retention time of the crystallized diammonium salt* of 6059-S free

*This compound was first crystallized by Dr. K.S. Yang of Lilly Res. Lab., Eli Lilly & Co., Indianapolis, IN, U.S.A.

acid, the structure of which was determined to be that of the *R*-epimer by X-ray diffraction [6].

The pure *R*- or *S*-epimer obtained by preparative chromatography was re-chromatographed immediately under the same conditions and gave only one peak. This result indicates that the ratios of the *R*- to the *S*-epimer do not change and no decomposition product is produced during this chromatographic procedure. On the UV spectra, no difference in absorbance between the *R*- and *S*-epimers was observed at any wavelength. Accordingly, the ratios of the peak areas of the *R*- to the *S*-epimer represent the ratios of their contents.

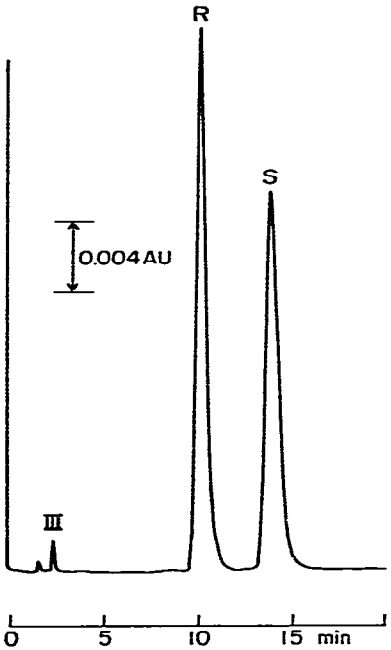

Fig. 2. Determination of the ratio of the *R*- to the *S*-epimer in a pharmaceutical material. Conditions as in text (eluent A); R, *R*-epimer; S, *S*-epimer; III, 1-methyl-1H-tetrazole-5-thiol.

Quantitative determination of 6059-S

Decarboxy-6059-S (II), one of the degradation products, was not eluted under isocratic conditions using eluent A as described under Experimental. Information about this compound, however, is important for the determination of the purity of pharmaceutical raw materials and commercial products and the metabolites in biological materials, because it has some antibacterial activity [7].

The compound 6059-S was eluted at a convenient time under the chromatographic conditions using eluent B, although the separation of the epimers was incomplete. The chromatography allows adequate elution of decarboxy-6059-S (II) without overlapping by the internal standard, *o*- or *p*-acetanisidide.

An example of the chromatogram is shown in Fig. 3. The eluates corresponding to the two 6059-S peaks were fractionated and each fraction was re-chromatographed immediately under the same conditions. No extra peaks were observed on the chromatogram. From this result, decomposition of 6059-S during the chromatographic procedure can be neglected. Two peaks of compounds II and III observed in Fig. 3 arose from impurities in the sample material itself.

The determination of 6059-S was based on the ratio of the combined peak area of the epimers to the peak area of the internal standard measured by an integrator. The calibration graphs obtained by this method showed good linearity in the range 0.5—0.1 mg/ml. We confirmed that mannitol or glucose added to the pharmaceutical preparation did not interfere with the determination of 6059-S.

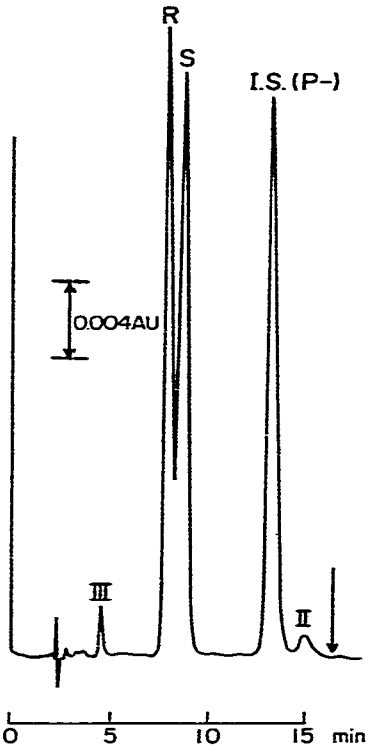

Fig. 3. Determination of 6059-S in a pharmaceutical material. Conditions as in text (eluent B; 25% methanol); volume injected; 5 µl; 0.4 mg/ml; R, *R*-epimer; S, *S*-epimer; II, decarboxy-6059-S; III, 1-methyl-1H-tetrazole-5-thiol; I.S.(P-), *p*-acetanisidide; arrow, *o*-acetanisidide (I.S.) to be eluted.

Determination of 6059-S in human urine

Chromatography using eluents A and B was tested for determining 6059-S in human urine. Interfering absorptions at the retention times of the 6059-S epimers were found in the chromatogram of control urine using eluent A or methanol-free eluent A. Fig. 4 shows a typical chromatogram obtained using

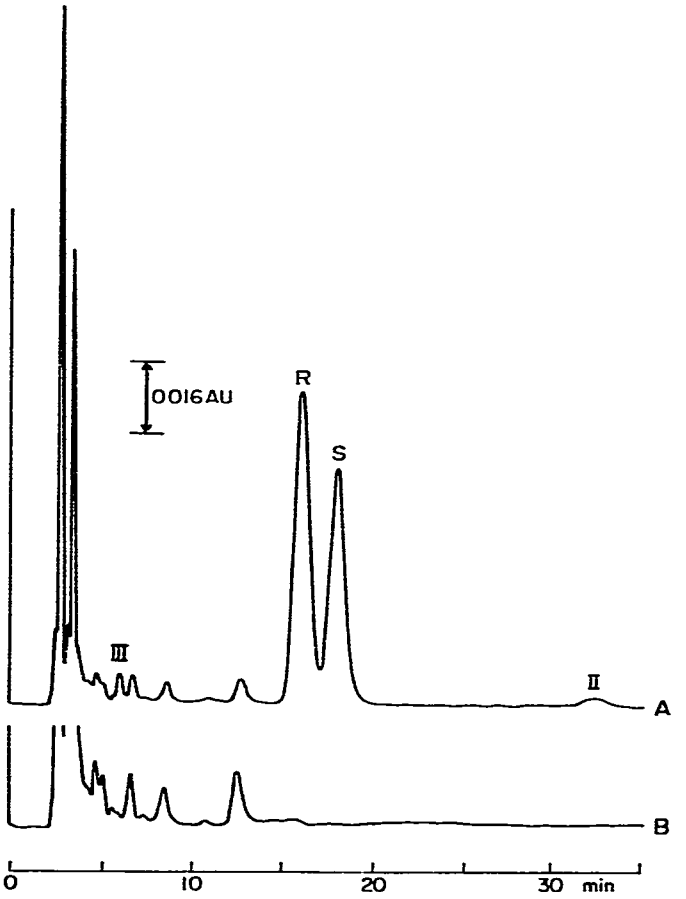

Fig. 4. (A) Chromatogram of human urine excreted following administration of 6059-S. Conditions as in text (eluent B; 20% methanol); volume injected; 3 μl; R, *R*-epimer; S, *S*-epimer; II, decarboxy-6059-S; III, 1-methyl-1H-tetrazole-5-thiol; *R* + *S*, 3.2 mg/ml. (B) Chromatogram of control human urine.

eluent B with a methanol content of 20% for a sample of urine containing 3 mg/ml of 6059-S from a volunteer. The front and rear peaks of 6059-S corresponding to compounds III and II, respectively, originate from the dosed material. During the time-course assay experiment, the amounts of both compounds in urine remained almost constant. In the experiment employing spiked urine samples, the recovery in the clean-up procedure by use of the Sep-Pak C_{18} cartridge proved to be quantitative, and the detection limit was about 0.01 mg/ml.

Table I gives the results for the amounts of 6059-S excreted in the urine of seven volunteers, determined by HPLC, together with the bioassay data and for the ratios of the *R*- to the *S*-epimer obtained by HPLC. The average accumulated excretion recoveries up to 12 h for the T group and up to 8 h for the F group were 795.7 and 782.3 mg, respectively. The actual average amounts of 6059-S injected were determined to be 975 mg from analysis of the residue in an injector and a bottle. Consequently, the corrected average

TABLE I

EXCRETED AMOUNTS AND RATIO OF *R*- AND *S*-EPIMERS OF 6059-S IN URINE FOLLOWING A SINGLE INTRAVENOUS ADMINISTRATION OF 1 g TO VOLUNTEERS

Dosage *R/S* ratio: T, 1.11; F, 1.05.

Group	Subject	Result	Time after administration (h)						
			0—1	1—2	2—4	4—6	6—8	8—12	Total*
T	M.O.	HPLC (mg)	338	152	152	71	38	29	780
		Bioassay (mg)	365	153	146	68	36	29	797
		R/S ratio	1.35	1.08	1.07	0.94	0.90	0.90	1.15
	S.O.	HPLC	311	168	152	74	44	35	784
		Bioassay	364	164	151	71	40	32	822
		R/S ratio	1.35	1.13	1.07	1.01	0.97	0.95	1.17
	M.H.	HPLC	363	175	163	68	31	23	823
		Bioassay	364	172	164	63	30	24	817
		R/S ratio	1.26	1.11	1.02	0.94	0.89	0.90	1.12
F	H.T.	HPLC	311	167	185	93	44		800
		Bioassay	337	194	186	81	35		833
		R/S ratio	1.27	1.16	1.03	0.97	0.96		1.14
	H.I.	HPLC	323	172	182	88	43		808
		Bioassay	354	173	178	84	40		829
		R/S ratio	1.18	1.06	1.08	0.95	0.98		1.09
	M.K.	HPLC	296	160	178	97	45		776
		Bioassay	314	167	173	83	42		779
		R/S ratio	1.12	1.08	1.01	1.01	0.98		1.06
	S.M.	HPLC	302	146	169	76	52		745
		Bioassay	305	154	184	85	56		784
		R/S ratio	1.16	1.10	1.06	0.98	1.05		1.10

*T, 0—12 h; F, 0—8 h.

recoveries were 81.6% for the T group and 80.2% for the F group. The excretion rate—time curves are given in Fig. 5. One-compartment model analysis of the time-course data revealed that 6059-S dosed intravenously was excreted in urine in the intact form with a rate constant of $0.433 \pm 0.047\ h^{-1}$ ($n = 7$).

In Table I, it is noteworthy that the ratios of the *R*- to the *S*-epimer changed in the time-course of excretion. The early ratios were higher than that in the dosed 6059-S drug itself, but decreased with time to become lower than that of the dosed drug. The spiked human urine samples (pH 7.0), with initial ratios of the *R*- to the *S*-epimer of 1.27 and 0.71, were allowed to stand for 2 h at 37°C in order to examine whether interconversion between the two epimers occurs in the bladder. As both ratios remained almost constant with time, we can regard the ratios determined on collected urine samples as true values for excretion. The fact that the ratios of the *R*- to the *S*-epimer in total urine approached the level in the dosed drugs suggests that no interconversion between the two epimers occurs in the human body. Recently, Yamada et al. [8] reported that the faster excretion of the *R*-

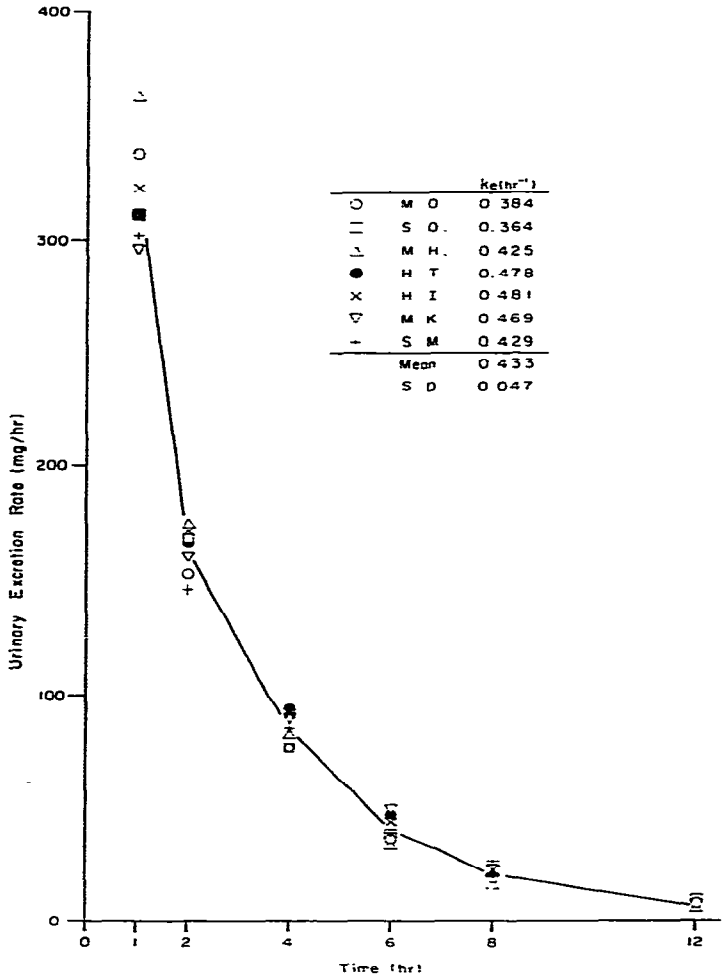

Fig. 5. Urinary excretion rate of 6059-S after intravenous administration of 1 g of 6059-S.

epimer compared with that of the *S*-epimer can be explained by the larger unbound fraction of the former in human plasma.

Correlation with microbiological assay

Fig. 6 shows the correlation of HPLC and microbiological data in Table I for the amounts of 6059-S excreted. Linear regression analysis led to the equation $y = 1.085x - 8.57$, with a correlation coefficient of 0.9954 and a coefficient of variation of 7.1%. The slope of greater than unity and the negative intercept mean that the microbiological results are higher than the HPLC results in the early stages and vice versa in the later stages. As the ratio of the *R*- to the *S*-epimer in the standard solution of 6059-S is equivalent to the dosage level and the *R*-epimer is approximately three times more active than the *S*-epimer against the test organism [9], the estimated concentrations of 6059-S in the samples tend to be higher by microbiological assay than by

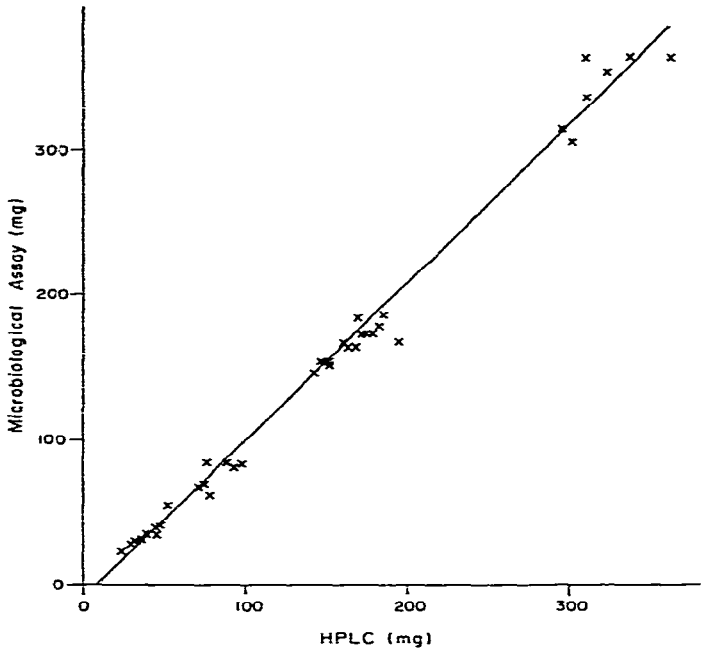

Fig. 6. Linear regression analysis of amounts of 6059-S in human urine samples determined by HPLC and microbiological assay.

HPLC when the ratio of the *R*- to the *S*-epimer in the sample is higher than the dosage level, namely in the early stages. This may explain the discrepancy between the values obtained by the two assay methods and the characteristics of the regression analysis.

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