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High-performance liquid chromatographic determination of (*S*)-(−)-ofloxacin and its metabolites in serum and urine using a solid-phase clean-up

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ABSTRACT

A sensitive and selective method for the simultaneous determination of (*S*)-(−)-ofloxacin [(*S*)-(−)-OFLX] and its metabolites in serum and urine was developed using isocratic high-performance liquid chromatography with a specific solid-phase extraction procedure. (*S*)-(−)-OFLX and its metabolites, desmethyl-(*S*)-(−)-OFLX and (*S*)-(−)-OFLX N-oxide, were eluted from a C_8 solid-phase column with recoveries of more than 98%. These compounds were separated and determined by means of a reversed-phase column with fluorimetric detection. Validation studies showed that the results were linear for (*S*)-(−)-OFLX in serum over the range 10–1200 ng/ml and in urine over the range 1–200 μg/ml. Analysis for (*S*)-(−)-OFLX and its metabolites showed good precision and accuracy with a relative standard deviation of less than 6%.

INTRODUCTION

(*S*)-(−)-Ofloxacin [(*S*)-(−)-OFLX](I), full name (*S*)-(−)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid hemihydrate, is a new quinolone antibacterial agent and the (*S*)-(−)-enantiomer of racemic ofloxacin [(±)-OFLX]. Compound I was 8–128 times more potent in inhibiting the multiplication of Gram-positive and Gram-negative bacteria than (*R*)-(+)-OFLX, and approximately twice as active as the racemate, (±)-OFLX [1,2].

Recently, we reported the stereoselective disposition of (±)-OFLX in rats [3]. Marked differences existed between the enantiomers in two pharmacokinetic parameters, namely the half-lives and areas under the curve (AUC), those of (*R*)-(+)-OFLX both being greater than those of I. Enantiomeric differences were also seen in the biliary excretion of OFLX glucuronide caused by the stereoselective metabolism in the hepatic microsomal enzymes [4]. Further, it has been elucidated that I undergoes only limited metabolism to form its metabolites, desmethyl-(*S*)-(−)-OFLX (II) and (*S*)-(−)-OFLX N-oxide (III), in experimental animals.

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Consequently, it is important to clarify the pharmacokinetics of I and its metabolites in humans for the sake of safety and efficacy in clinical use.

Many methods for the determination of (±)-OFLX in biological fluids by high-performance liquid chromatography (HPLC) have been reported [5–10]. Since I is an enantiomer of (±)-OFLX, the application of methods suitable for the latter are useful in the determination of the former. However, efficient separation of the unchanged drug and its metabolites has not been achieved by isocratic HPLC [9,10], and the sensitivity and selectivity of these methods are poor because high recoveries of the compounds, especially of the metabolites, are not yielded by the liquid–liquid extraction commonly used for the extraction of quinolone antibacterials.

The purpose of this study was to develop a sensitive and selective method for the separation and determination of I and its metabolites in serum and urine by isocratic HPLC. Solid-phase extraction was used as a sample clean-up procedure and gave a complete recovery of I and its metabolites in serum and urine, avoiding the interference peaks on the chromatograms derived from the biological fluids. These compounds were completely separated by reversed-phase chromatography with fluorimetric detection. The proposed method can be successfully applied to pharmacokinetic studies in humans.

EXPERIMENTAL

Materials

Compounds I, II, III and the internal standard (I.S.), with structures as shown in Fig. 1, were synthesized in this laboratory. HPLC-grade tetrahydrofuran (Kanto Chemical, Tokyo, Japan) was used for the mobile phase. Water was obtained from a Milli-Q water purification system (Millipore, Milord, MA,

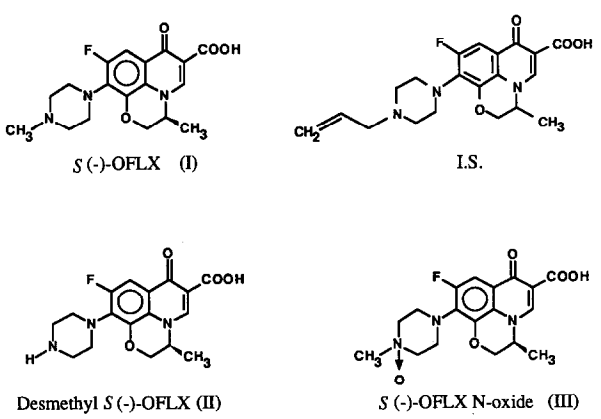

Fig. 1. Structures of (*S*)-(−)-OFLX, desmethyl-(*S*)-(−)-OFLX, (*S*)-(−)-OFLX N-oxide and the internal standard (I.S.).

U.S.A.). Chloroform (HPLC grade) was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents and chemicals were of analytical-reagent grade unless specified otherwise.

HPLC apparatus

The chromatographic system consisted of an HPLC pump (Model CCPM; Tosoh, Tokyo, Japan), a fluorescence detector (Model F-1150; Hitachi, Tokyo, Japan) and a sample autoinjector with a 50-μl fixed loop (Model AS-8000; Tosoh). Data integration was performed on a Maxima 820 Chromatography workstation (Millipore).

HPLC conditions

A TSKgel ODS-80T$_M$ column (150 mm × 4.6 mm I.D., Tosoh) was used for the analysis of serum and urine samples. Compounds I, II, III and the I.S. were eluted isocratically using a mobile phase consisting of tetrahydrofuran–50 m*M* potassium dihydrogenphosphate (pH 2.0, adjusted with orthophosphoric acid)–1 *M* ammonium acetate (7.5:92.5:1, v/v). The mobile phase was degassed in an ultrasonic bath prior to use. Detection was performed by spectrofluorimetry at an excitation wavelength of 296 nm and an emission wavelength of 504 nm. The flow-rate was 1 ml/min. All analyses were carried out at ambient temperature.

Solid-phase extraction

Bond Elut columns, which were packed with 200 mg of C_8 bonded phase (Analytichem International, Harbor City, CA, U.S.A.), were placed in a Vac Elut manifold (Analytichem International) and conditioned by washing with 4 ml of methanol followed by 8 ml of distilled water and 8 ml of 50 m*M* potassium dihydrogenphosphate. The serum samples (0.1 ml) were diluted with 0.1 ml of 50 m*M* potassium dihydrogenphosphate containing 213 and 20.4 ng/ml I.S. for the determination of I and its metabolites, respectively. The samples were then loaded on the columns and drawn through by means of the vacuum manifold. The flow-rate was kept constant by controlling the vacuum (755 mmHg). The column was washed again with 8 ml of 50 m*M* potassium dihydrogenphosphate followed by 4 ml of tetrahydrofuran–distilled water (2:8, v/v) and allowed to drain completely. Compounds I, II, III and the I.S. were eluted with 2 ml of tetrahydrofuran–0.15% orthophosphoric acid (2:8, v/v). The eluate was evaporated to dryness in a evaporator (EC-57C; Sakuma Seisakusyo, Tokyo, Japan). For HPLC analysis, the residue was dissolved in 200 μl of tetrahydrofuran–distilled water (8:92, v/v) by ultrasonic treatment and 50 μl of the solution were injected into the chromatograph. Urine samples were treated using the same procedures as for the serum samples, except for the addition of I.S. concentrations of 25 μg/ml and 20.4 ng/ml for the determination of I and its metabolites, respectively.

Liquid–liquid extraction

To serum (0.2 ml) or urine (0.2 ml) samples was added 1 ml of 0.1 *M* phosphate buffer (pH 7.0) containing the I.S. at the same concentrations as for the solid-phase extraction procedure. After the addition of 5 ml of chloroform, the tubes were stoppered and agitated on a rotary mixer for 10 min. The tubes were subsequently centrifuged at 1500 *g* for 10 min at 4°C to separate the layers. After the aqueous layer had been removed with an aspirator, 4 ml of the chloroform layer were transferred to a fresh glass tube and evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 200 μl of the mobile phase and 50 μl of the solution were applied to the HPLC system.

Recovery study

Extraction recoveries ($n = 10$ for solid-phase extraction, $n = 4$ for liquid–liquid extraction) were determined by comparing the peak-area ratios of chromatograms obtained from extracted serum and urine samples by solid-phase and liquid–liquid extraction methods with those of stock standard solutions containing 1 μg/ml of I, II and III.

Detection limit

The detection limit, based on the standard deviations of the serum and urinary concentrations of these compounds and the noise level, was obtained by the following equation, as described elsewhere [11]:

$$\text{Detection limit} = \text{noise level} + \frac{3\,(\text{S.D.}_1 + \text{S.D.}_2 + \cdots + \text{S.D.}_n)}{n}$$

where the noise level is the mean value of the blank signal, S.D. is the standard deviation of an individual concentration range assayed and n is the number of concentration ranges assayed.

Standard preparation

Stock standard solutions of I, II and III were prepared by dissolving *ca.* 100 mg of each compound in 100 ml of distilled water. That of the I.S. (1 mg/ml) was also prepared in distilled water after dissolution in a small amount of 0.15% orthophosphoric acid. Working standard solutions were prepared from these stock standard solutions by sequential dilution with distilled water for HPLC calibration. The stock solutions were stored at 4°C when not in use. The solutions were stable in the dark at 4°C for at least three months.

Validation study

Standard serum and urine were prepared by adding aliquots of the working standard solution to drug-free human serum and control urine. Standard serum solutions containing 10–1200 ng/ml I and 0.5–50 ng/ml metabolites and standard

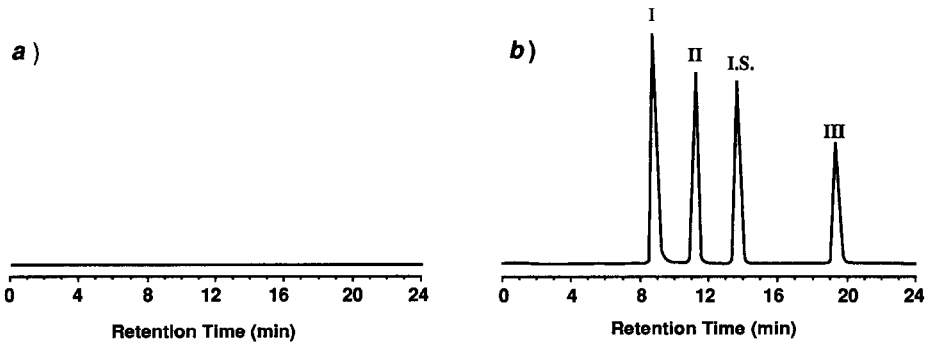

Fig. 2. Typical chromatograms of (a) drug-free human serum and (b) the same serum sample spiked with 1.16 μg/ml (*S*)-(−)-OFLX (I), 1.18 μg/ml desmethyl-(*S*)-(−)-OFLX (II), 1.13 μg/ml internal standard (I.S.) and 1.02 μg/ml (*S*)-(−)-OFLX N-oxide (III). The analytical conditions are described in the text.

urine solutions containing 1–200 μg/ml I and 0.5–50 ng/ml metabolites were prepared for calibration measurements. Serum and urine spikes for the intra-day and inter-day studies of the accuracy and precision of the assay (n = 7) were prepared at concentrations of 50.0 ng/ml and of 5.0 μg/ml for I, 11.8 ng/ml for II and 11.3 ng/ml for III.

RESULTS AND DISCUSSION

HPLC conditions

After characterization of the absorption spectra of I, its metabolites and the I.S. in the mobile phase, detection was performed with excitation at 296 nm and emission at 504 nm, which are the common maximum wavelengths of these compounds.

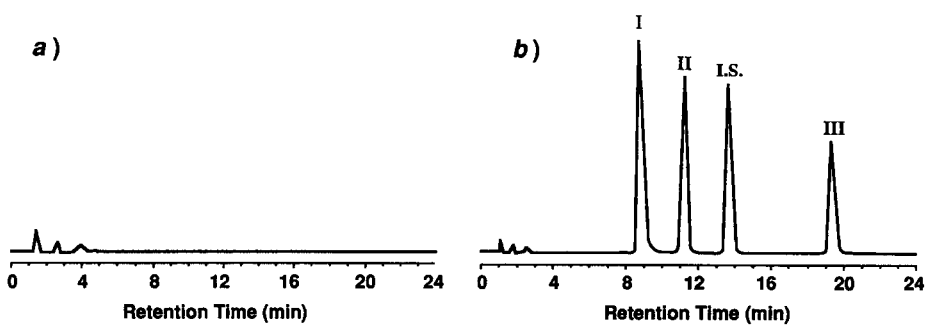

Fig. 3. Typical chromatograms of (a) control human urine and (b) the same urine sample spiked with 1.16 μg/ml (*S*)-(−)-OFLX (I), 1.18 μg/ml desmethyl-(*S*)-(−)-OFLX (II), 1.13 μg/ml internal standard (I.S.) and 1.02 μg/ml (*S*)-(−)-OFLX N-oxide (III). The analytical conditions are described in the text.

Several stationary and mobile phases were investigated to establish the optimum separation of these compounds. Acetonitrile has been used as the mobile phase for the quantitation of OFLX, as has methanol [5,7–9]. However, an efficient separation of I, II and III was not achieved with mobile phases containing acetonitrile or methanol. The addition of tetrahydrofuran to the mobile phase reduced the peak tailing and gave a good separation of these compounds with the addition of ammonium acetate.

Figs. 2a and 3a show chromatograms of a drug-free serum sample from a normal subject and of control urine, respectively, and Figs. 2b and 3b show chromatograms of the same serum and urine spiked with *ca.* 1 μg/ml of each compound. The chromatograms from blank serum and urine were free from interferences at the retention times corresponding to the drug peaks. Under the HPLC conditions employed, I, II, III and the I.S. were well resolved with retention times of 9.6, 11.3, 20.5 and 13.5 min, respectively. However, attention should be paid to the tetrahydrofuran concentration in the mobile phase, because changes in concentration substantially affect the retention times of these compounds. Many reports concerning the determination of (±)-OFLX by HPLC have been published. However, the separation between unchanged OFLX and its desmethyl form is still poor [9,10]. Few published methods have determined the retention characteristics of metabolites. The main purpose of this work was to establish a system for the complete separation of I and II. A typical chromatogram obtained from serum sample is shown in Fig. 4. As can be seen, there was complete baseline separation of I and II although the drug levels were very different. The resolution factor (R_s) between I and II in this system was 2.0, indicating efficient separation of the peaks. We also studied the influence of counter ions (pentane-, hexane- and octane-1-sulphonic acid and tetrabutylammonium hydrogensulphate) on the separation. The retention times of these compounds were prolonged with increasing counter-ion concentration, although the selectivity was still unchanged. Conse-

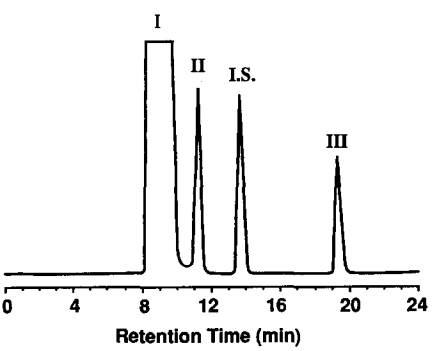

Fig. 4. Typical chromatogram of a serum sample from a volunteer. The serum sample was taken 30 min after an oral dose of 100 mg of (*S*)-(−)-OFLX. Peaks: I = (*S*)-(−)-OFLX; II = desmethyl-(*S*)-(−)-OFLX; I.S. = internal standard; III = (*S*)-(−)-OFLX N-oxide.

quently, ion-pair chromatography was found to be unnecessary for the separation of the unchanged drug and its metabolites.

Evaluation of solid-phase and liquid–liquid extraction procedures

The recoveries of I, II, III and the I.S. were investigated by solid-phase and liquid–liquid extraction methods. Fig. 5 compares the extraction percentages of these compounds by the two methods. Compounds I and II were extracted from the serum with chloroform at pH 7.0 with recoveries of 39.5 and 54.7%, respectively. pH 7.0 was the most suitable for the extraction of these compounds because the isoelectric point of I is *ca.* pH 7. The extraction percentages of III with chloroform were less than 5% even under optimum conditions. In order to obtain satisfactory extraction recoveries of quinolone metabolites, an attempt was made to precipitate serum protein with trichloroacetic acid, acetonitrile or methanol [10,12]. Although acceptable recoveries of the drugs were obtained, the chromatograms displayed many impurity peaks [9,10]. In contrast, the solid-phase extraction method gave good recoveries of more than 98% for all compounds. Moreover, as shown in Figs. 2 and 3, there were few, if any, interference peaks in drug-free serum and control urine. Using this extraction and purification method, a sensitive and selective determination of these compounds, especially of III, is possible.

Detection limits

The detection limits were determined according to the equation given under Experimental. Fig. 6 shows the chromatogram obtained by spiking with *ca.* 1

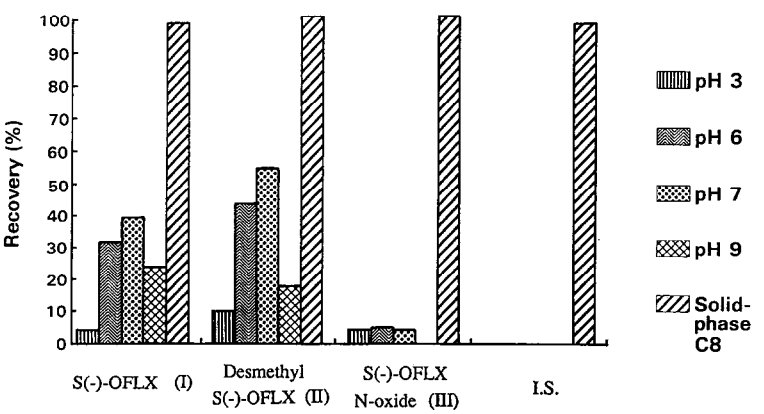

Fig. 5. Comparison of recoveries using liquid–liquid extraction with chloroform and solid-phase extraction of (*S*)-(−)-OFLX, its metabolites and the internal standard. (*S*)-(−)-OFLX N-oxide in phosphate buffer at pH 9.0 was not extracted with chloroform. The chloroform extraction of the internal standard was not tested.

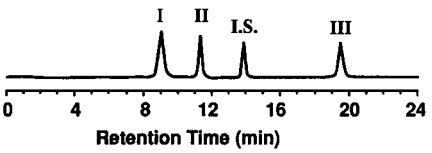

Fig. 6. Typical chromatogram of serum spiked with *ca.* 1 ng/ml (*S*)-(−)-OFLX (I), desmethyl-(*S*)-(−)-OFLX (II), (*S*)-(−)-OFLX N-oxide (III) and the internal standard (I.S.).

TABLE I

PRECISION AND ACCURACY OF DETERMINATION OF (*S*)-(−)-OFLX IN SPIKED HUMAN SERUM AND URINE

The spiked concentrations were 50.0 ng/ml (serum) and 5.0 μg/ml (urine). Each value represents the mean ± standard deviation for seven determinations.

Day of evaluation	Serum		Urine	
	Concentration found (ng/ml)	Relative standard deviation (%)	Concentration found (μg/ml)	Relative standard deviation (%)
1	51.64 ± 1.21	2.34	4.79 ± 0.00	0.06
2	50.87 ± 1.39	2.73	5.24 ± 0.01	0.15
3	46.69 ± 1.73	3.70	5.00 ± 0.02	0.30
4	51.10 ± 0.52	1.01	4.93 ± 0.01	0.20
Mean	50.08 ± 2.28	4.55	4.99 ± 0.19	3.74

TABLE II

PRECISION AND ACCURACY OF DETERMINATION OF DESMETHYL-(*S*)-(−)-OFLX IN SPIKED HUMAN SERUM AND URINE

The spiked concentrations were both 11.8 ng/ml (serum and urine). Each value represents the mean ± standard deviation for seven determinations.

Day of evaluation	Serum		Urine	
	Concentration found (ng/ml)	Relative standard deviation (%)	Concentration found (ng/ml)	Relative standard deviation (%)
1	11.65 ± 0.54	4.67	10.96 ± 0.63	5.71
2	10.73 ± 0.29	2.67	11.50 ± 0.67	5.84
3	10.87 ± 0.54	4.96	11.37 ± 0.50	4.41
4	11.20 ± 0.46	4.11	11.44 ± 0.50	4.38
Mean	11.11 ± 0.41	3.69	11.32 ± 0.24	2.14

TABLE III

PRECISION AND ACCURACY OF DETERMINATION OF (*S*)-(−)-OFLX N-OXIDE IN SPIKED HUMAN SERUM AND URINE

The spiked concentrations were both 11.3 ng/ml (serum and urine). Each value represents the mean ± standard deviation for seven determinations.

Day of evaluation	Serum		Urine	
	Concentration found (ng/ml)	Relative standard deviation (%)	Concentration found (ng/ml)	Relative standard deviation (%)
1	10.62 ± 0.55	5.19	10.94 ± 0.44	4.05
2	11.40 ± 0.48	4.18	10.51 ± 0.55	5.19
3	11.22 ± 0.54	4.82	11.30 ± 0.61	5.41
4	10.77 ± 0.52	4.87	11.11 ± 0.53	4.76
Mean	11.01 ± 0.37	3.34	10.97 ± 0.34	3.06

ng/ml I, II, III and the I.S. All the compounds can be detected adequately at a concentration using 1 ng/ml by fluorimetric detection. The standard deviation of the blank and of the samples containing the smallest added amount of I, II and III for the calibration graph (0–116 ng/ml) was used to calculate the detection limits for the serum and urine samples. These limits were found to be 0.86, 0.61 and 0.92 ng/ml in serum and 0.91, 0.91 and 1.06 ng/ml in urine for I, II and III, respectively. The limit of determination was the lowest concentration in the spiking experiment that was greater than the detection limit (10 ng/ml for I and 1 ng/ml for metabolites in serum, 1 μg/ml for I and 1 ng/ml for metabolites in urine).

Assay validation

The calibration graphs obtained on four consecutive days showed a linear relationship between the peak-area ratio of I or its metabolites and the I.S., with correlation coefficients for three analyses of more than 0.999 in the ranges 10–1200 ng/ml (serum) and 1–200 μg/ml (urine) for I, and there was excellent linearity in the range 0.5–50 ng/ml (serum and urine) for metabolites when this method was used. Typical intra- and inter-day reproducibility data are given in Tables I–III. The intra-assay relative standard deviations for the assays of I (50.0 ng/ml), II (11.8 ng/ml) and III (11.3 ng/ml) in spiked serum were less than 6%. In addition, acceptable accuracy of more than 90% was achieved at all concentrations examined. Good reproducibility of the method was indicated when replicate determinations were made on four consecutive occasions in serum at 50.0, 11.8 and 11.3 ng/ml for I, II and III, respectively.

Application to biological samples

The method was applied to a pharmacokinetic study of a human subject. Fig.

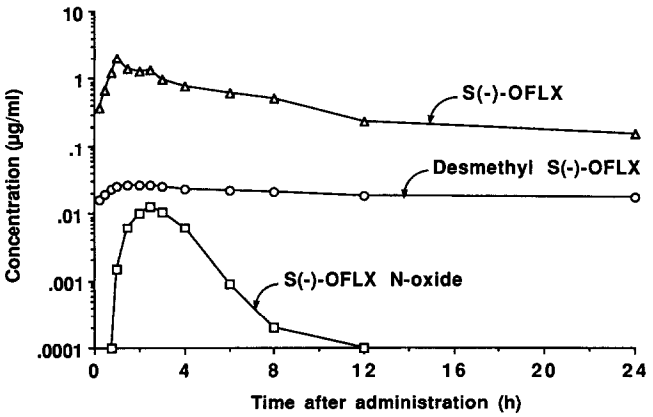

Fig. 7. Serum concentration–time profile of (*S*)-(−)-OFLX and its metabolites after a single 100-mg oral dose of (*S*)-(−)-OFLX to a human subject.

7 shows the serum concentration *versus* time profile of I and its metabolites after a single 100-mg oral dose of I to a volunteer. An unchanged serum drug concentration of 2.06 μg/ml was attained 1 h after the administration and thereafter it declined gradually. The peak serum concentrations of II and III were 26.4 and 12.4 ng/ml, respectively, and these concentrations were *ca.* 50–100 times lower than those of the unchanged drug. The present method could be applied to pharmacokinetic studies not only of I but also its metabolites.

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