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Note

Simplified high-performance liquid chromatographic method for determination of mizoribine in human serum

HIDEO HOSOTSUBO*

Central Laboratory for Clinical Investigation, Osaka University Hospital, 1-1-50, Fukushima-ku, Osaka 553 (Japan)

SHIROU TAKAHARA

Department of Urology, Osaka University Hospital, 1-1-50, Fukushima-ku, Osaka 553 (Japan)

and

NOBUYUKI TAENAKA

Intensive Care Unit, Osaka University Hospital, 1-1-50, Fukushima-ku, Osaka 553 (Japan)

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Mizoribine (Bredinin; 4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) is a novel imidazole nucleoside compound (Fig. 1) with immunosuppressive activity, which was isolated in 1974 from the culture filtrate of *Eupenicillium brefeldianium* M-2166 [1]. The immunosuppressive effect of mizoribine may be mediated at the subcellular level through the inhibition of nucleic acid synthesis. The immunosuppressive action of mizoribine is comparable with that of azathioprine, but mizoribine is known to be free of the deleterious side-effects of azathioprine, such as bone marow suppression and hepatotoxicity [2].

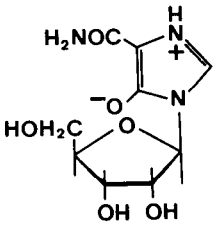

Fig. 1. Structure of mizoribine.

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The importance of monitoring the serum mizoribine level to optimize the therapeutic benefit and to avoid toxicity has been noted previously [3]. Since 80–90% of mizoribine is excreted unchanged in the urine [4], it is desirable to be able to determine rapidly the serum mizoribine level when renal function is compromised, either directly owing to rejection or secondarily owing to drug therapy, as is often the case with cyclosporin A.

Until recently, the only method available for determining the serum mizoribine level has been a high-performance liquid chromatographic (HPLC) method [5]. Although this method has proved to be reliable in routine, straightforward, clinical situations, it has limitations when rapid clinical feedback of the mizoribine level is required.

We have developed a new HPLC method, which is rapid, selective and reproducible. Its usefulness was investigated by measuring the drug levels in serum samples obtained during pharmacokinetic studies in renal transplant patients.

EXPERIMENTAL

Chemicals

Mizoribine was a gift from Toyo Jozo (Shizuoka, Japan). All other chemicals were of analytical-reagent grade.

Chromatography

Chromatographic equipment was obtained from Shimadzu (Kyoto, Japan). An LC-6A liquid chromatograph equipped with a Rheodyne 7125 sample injector was connected with a SPD-6A variable-wavelength UV detector and a C-R4A computing integrator. The volume of the injection loop was 50 μl. A stainless-steel column (15 cm$\times$6.0 mm I.D.) packed with 5-μm CLC-NH_2 (Shimadzu) was used at room temperature. The mobile phase was 70 m*M* potassium dihydrogenphosphate (pH 2.5)–acetonitrile (30:70), delivered at a flow-rate of 1.0 ml/min. The column effluent was monitored at 280 nm at a sensitivity of 0.005 a.u.f.s.

Sample preparation

Acetonitrile (0.1 ml) was added to 0.1 ml of serum. The tube was vigorously shaken for 30 s, and after 5 min equilibration it was shaken again for 30 s. After centrifugation (2 min at 12 000 *g*), a 50-μl aliquot of the clear supernatant was injected into the HPLC system.

Quantitation

A stock solution of mizoribine was prepared in water at a concentration of 100 μg/ml. Using the mizoribine standard solutions, separate series of drug-free human serum samples were spiked with concentrations ranging from 0.25 to 10.0 μg/ml. These calibration samples were extracted as described above.

Application to human subjects

Two renal transplant patients were administered oral doses of 50 and 100 mg of mizoribine. Blood samples were drawn before and at various times after admin-

istration. Samples were centrifuged to obtain serum fractions, which were stored at −20 °C until analysis.

RESULTS AND DISCUSSION

Chromatographic separation

The isocratic elution and 1.0 ml/min flow-rate gave a retention time of 9.2 min for mizoribine. Fig. 2 shows typical chromatograms obtained for a drug-free human serum, serum with 1.0 μg/ml mizoribine added and a clinical sample containing 0.88 μg/ml mizoribine. The chromatographic analysis of one sample required less than 12 min. From the serum chromatograms, it is clear that the mizoribine peak did not interfere with endogenous serum constituents.

Calibration curve

There is a linear relation between the peak height and the concentration of mizoribine in the standard serum samples ($r=0.999$), up to 10.0 μg/ml mizoribine. The limit of quantitation of the assay (i.e. peak height corresponding to twice the baseline noise) was found to be 0.01 μg/ml.

Recovery and stability

Known amounts of mizoribine were added to drug-free serum to provide concentrations ranging from 0.25 to 2.0 μg/ml. After six replicate extractions and chromatography of five samples of each concentration, the peak heights obtained were compared with the peak heights obtained for standard serum concentrations. Absolute recovery was calculated as the amount of mizoribine measured divided by the amount of mizoribine added (μg/ml) × 100. The results are sum-

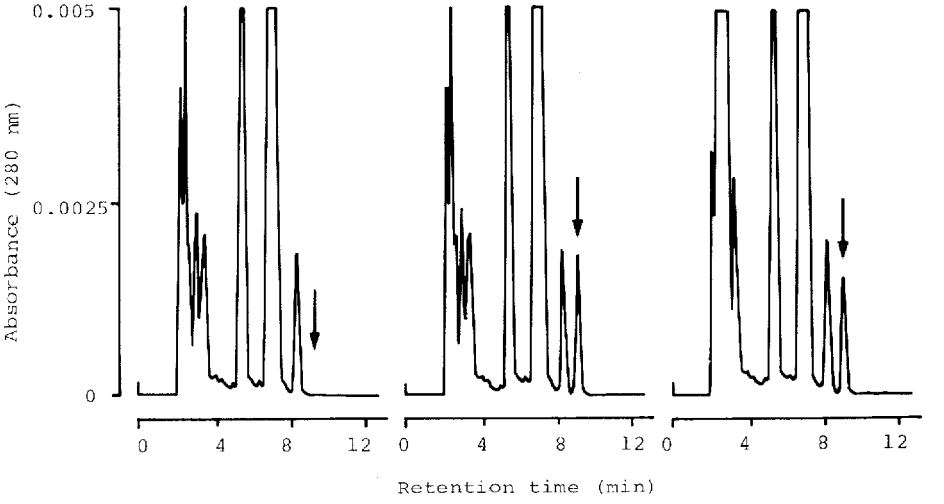

Fig. 2. Chromatograms of (A) drug-free human serum, (B) human serum containing 1.0 μg/ml mizoribine (arrow), and (C) serum collected from renal transplant patient after an oral dose of mizoribine (calculated concentration 0.88 μg/ml).

TABLE I

RECOVERY OF MIZORIBINE FROM SERUM ($n=6$)

Mizoribine added (μg/ml)	Found (mean ± S.D.) (μg/ml)	Recovery (mean ± S.D.) (%)
0.25	0.240 ± 0.008	96.0 ± 3.36
0.50	0.497 ± 0.012	99.4 ± 2.49
1.00	0.958 ± 0.019	95.8 ± 1.92
1.50	1.452 ± 0.022	96.8 ± 1.45
2.00	1.920 ± 0.019	96.0 ± 0.96
Mean		96.8 ± 2.04

TABLE II

PRECISION FOR MIZORIBINE IN SERUM

Nominal concentration (μg/ml)	Within-day ($n=10$)			Between-day ($n=6$)		
	Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)
0.25	0.261	0.013	4.980	0.247	0.016	6.478
0.5	0.497	0.022	4.427	0.514	0.026	5.058
1.0	1.025	0.017	1.659	0.983	0.033	3.357
2.0	2.018	0.017	0.842	2.058	0.051	2.478
4.0	3.970	0.024	0.605	4.107	0.087	2.118
Mean			2.503			3.898

marized in Table I. A 95.8–99.4% absolute recovery of mizoribine is obtained in the concentration range 0.25–2.0 μg/ml of serum.

Serum samples from patients were stored for 6 months at $-20°$C and yielded concentration values identical with those obtained for the fresh serum sample.

Precision and accuracy

The precision and accuracy of the method were evaluated by repetitive analysis of five aliquots of drug-free pooled serum spiked with mizoribine at different concentrations. The results are shown in Table II. For the within-day analysis, the average coefficient of variation (C.V.) was 2.503%. Table II also shows the between-day precision of the assays (every other day over one week); the average C.V. was 3.898%.

Interference study

The interference with the mizoribine peak was studied for the following drugs: amikacin, amphotericin B, azathioprine, chloramphenicol, cimetidine, cyclosporin A, 5-fluorocytosine, methylprednisolone, miconazole and vancomycin. They were chosen for testing because at least twenty of the renal transplant patients ingesting mizoribine were ingesting these drugs simultaneously. None of

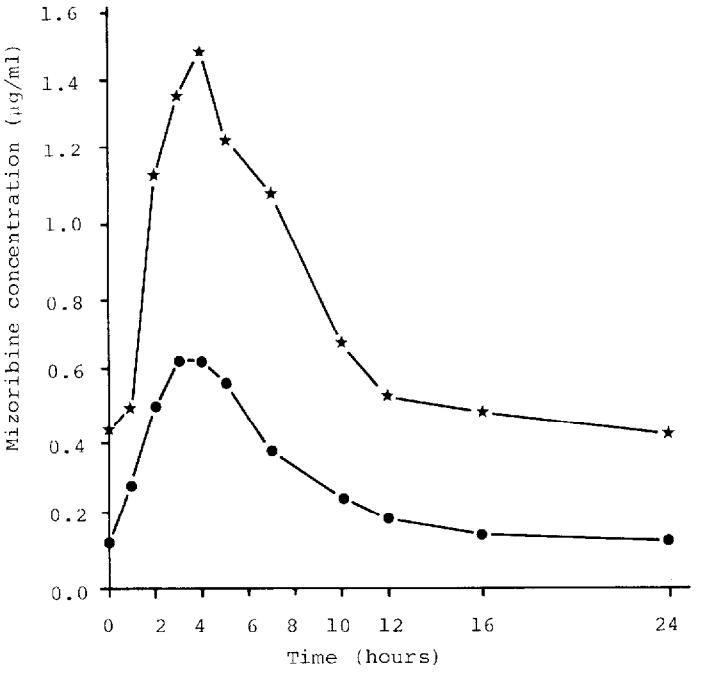

Fig. 3. Detection of mizoribine in serum of renal transplant patients after an oral dose: ● = 50 mg, patient A; ★ = 100 mg, patient B.

these ten drugs interfered with the mizoribine peak. They all eluted much earlier than the mizoribine peak under the chromatographic conditions applied.

Clinical applications

The method described here is an accurate and reproducible procedure for the measurement of mizoribine concentrations in human serum. Fig. 3 shows the serum concentration–time profiles of mizoribine for two renal transplant patients after an oral dose of 50 or 100 mg. Serum levels of mizoribine were observed to reach maxima of 0.63 and 1.45 μg/ml after 3 and 4 h in patients A and B, respectively.

CONCLUSION

The reported method has the necessary precision and selectivity for therapeutic monitoring of patients treated with mizoribine. In addition, the excellent sensitivity permits accurate determination of the pharmacokinetics of mizoribine following oral doses. This method allows for rapid, specific and sensitive quantitation of mizoribine in the serum. The described serum mizoribine extraction procedure is simple: a one-step precipitation of serum proteins with acetonitrile. The sample volume, extraction time and chromatographic time are all lower than in the existing method. Furthermore, baseline resolution of mizoribine and improved precision are obtained. With this procedure, the use of standard HPLC

techniques allows quantitation at concentrations suitable for pharmacokinetic studies and routine monitoring in organ transplantation.

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