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Note

Determination of mizoribine in plasma using ion-pair high-performance liquid chromatography

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Mizoribine (MZB), a nucleoside antibiotic produced by *Eupenicillium brefeldianum*, has received favorable attention as an immunosuppressive agent capable of prolonging canine and human renal allograft survival [1–4]. MZB is similar to the antimetabolite azathioprine in its mechanism of action and immunosuppressive potency [5], but does not possess hepatotoxic or myelosuppressive side-effects [2,4,6] MZB has replaced azathioprine in immunosuppressive protocols for clinical renal transplantation in Japan; however, a sensitive, rapid, and reproducible high-performance liquid chromatographic (HPLC) method which utilizes an internal standard (I S) has not been reported

Problems have arisen in developing a suitable method to analyze MZB due to the drug's hydrophilic nature and poor solubility in organic solvents Takada et al [7] developed a reversed-phase HPLC method which showed good sensitivity (0 25 μg/ml), but required a mobile phase consisting of 0 1 *M* imidazole and acetonitrile. The MZB manufacturer (Toyo Jozo, Tokyo, Japan) has

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described both an HPLC method, which requires a special column packing of octadecylsilane with covalently attached amino groups, as well as a less sensitive colorimetric assay [8].

Our laboratory has recently developed an ion-pair HPLC method with improved accuracy, precision, and sensitivity using an I.S The method has been used to determine MZB plasma levels in canine renal allograft recipients and may be used for human studies as well.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of an HP 1090A liquid chromatograph with a diode-array detector, automatic injector, heated column compartment, and Think-Jet printer (Hewlett-Packard, Palo Alto, CA, U S A) The separations were performed on an octadecylsilane column (Hypersil ODS, 5 μm particle size, 200 mm $\times$ 4 6 mm; Hewlett-Packard).

Chemicals

Perchloric acid (70–72%), potassium chloride, sodium hydroxide, hydrochloric acid, dibasic sodium phosphate (Curtin Matheson Scientific, Eden Prairie, MN, U.S A), octanesulfonic acid, and 3-methylxanthine (I S) (Sigma, St Louis, MO, U S A) were analytical grade Dichloromethane, methanol, and water (Mallinckrodt, Paris, KY, U S A) were HPLC grade Mizoribine was provided by Toyo Jozo

Drug solutions

Two solutions of MZB (100 and 50 μg/ml) were prepared in water A 1 mg/ml stock solution of the I S. was prepared in dilute sodium hydroxide An aliquot of the stock solution was added to water to a final concentration of 65 μg/ml

Chromatography

The mobile phase consisted of methanol–20 m*M* dibasic sodium phosphate (2 98, v/v), pH 3, containing octanesulfonic acid (0 04%, w/v) which was pumped through the column at 1.3 ml/min At 1 1 min the methanol content was increased by a one-step gradient to 6% to elute the I S. The column compartment was maintained at 40 °C MZB and the I S were detected at 275 nm (25 ma.u f s). A 2-μl volume was injected onto the column

Standard curves

A large standard calibration curve was prepared ($n = 4$ for each concentration point) in dog plasma on each of three separate days for computing linearity, precision, and accuracy The standard curves were constructed by adding

volumes of MZB to dog plasma to achieve drug concentrations ranging from 0 1 to 10 μg/ml

Quantification

Calibration curves were plotted using peak-height ratios of MZB to I S versus known MZB standard concentrations The concentrations of the quality controls (QCs) and dog samples were subsequently determined from the calibration curves

Sample preparation

Plasma samples were prepared by placing 500 μl into a clean 10 mm × 75 mm glass tube containing 50 μl of I.S The proteins were precipitated by adding 50 μl of perchloric acid The excess acid was removed with 50 μl of saturated potassium chloride The tubes were mixed well, centrifuged, and 350 μl of the supernatant were transferred to a clean glass tube Each sample was rendered alkaline with 35 μl of 10 *M* sodium hydroxide and extracted with 1 5 ml of dichloromethane Approximately 250 μl of the aqueous phase were removed, neutralized with 20 μl of 2 *M* hydrochloric acid, and transferred to HPLC vials for injection

Quality controls

QCs were analyzed in triplicate over three days to determine accuracy and between-run precision of MZB Drug-free dog plasma was prepared to contain MZB at three concentrations 0 25, 1 0, and 5 0 μl/ml The solutions were mixed, separated into 600-μl aliquots, and stored at −20°C Prior to analysis, three QCs from each concentration were brought to room temperature and prepared with the calibration curve samples

Statistics

Linearity was calculated by linear regression analysis and reported as r^2 The intercept and slope parameters include standard deviation (S D) estimates The coefficient of variation (C V) was determined by dividing the sample S D by the mean (y) and expressing the quotient as a percentage

RESULTS

Linearity, accuracy, and within-run precision

Data describing the linearity, within-run precision, and accuracy of one large calibration curve are shown in Table I The linearity was good over the concentration range studied (0 25–10 μg/ml) with an r^2 value of 0 9995, a slope of 0 2490 ± 0 0011 and an intercept of 0 0139 ± 0 0192 The within-run precision calculated from the QC samples analyzed with the calibration curve showed a C V of 8 9% at 0 25 μg/ml, 2 1% at 1 0 μg/ml, and 1 3% at 5 0 μg/ml

TABLE I

LINEARITY AND WITHIN-RUN PRECISION OF MIZORIBINE

$r^2 = 0\ 995$, slope $= 0\ 2490 \pm 0\ 0011$ (mean $\pm$ S D), intercept $= 0\ 0139 \pm 0\ 0192$ (mean $\pm$ S D)

Concentration added (μg/ml)	Concentration found (mean $\pm$ S D , $n=4$) (μg/ml)	C V (%)
0 25	0 29 $\pm$ 0 01	3 2
0 50	0 45 $\pm$ 0 04	8 1
1 0	0 99 $\pm$ 0 06	6 3
2 5	2 46 $\pm$ 0 04	1 5
5 0	5 02 $\pm$ 0 10	2 0
10 0	10 00 $\pm$ 0 15	1 4

TABLE II

BETWEEN-RUN PRECISION AND ACCURACY OF MIZORIBINE QUALITY CONTROLS

Concentration added (μg/ml)	Concentration found (mean $\pm$ S D , $n=9$) (μg/ml)	C V (%)
0 25	0 31 $\pm$ 0 05	14 8
1 0	1 06 $\pm$ 0 04	4 2
5 0	5 01 $\pm$ 0 16	3 3

Limits of quantitation and detection

The limit of quantitation was defined as the lowest calibration standard which would have a C V $<20\%$ A series of samples ($n=14$) were prepared at 0.1 μg/ml and analyzed with the other calibration standards over three days The C V was 27.7%, therefore a 0 1 μg/ml concentration was identified as the level of detection The next highest standard, 0 25 μg/ml, consistently had a C.V. $<20\%$ and was determined to be the limit of quantitation

Between-run precision and accuracy

The QC data representing the between-run precision and accuracy of determining MZB in plasma over a three-day period is shown in Table II The 0 25 μg/ml QC had a mean ($\pm$S.D.) value of 0 31 $\pm$ 0 04 μg/ml (C V $=14\ 8\%$), the 1 0 μg/ml QC was 1 05 $\pm$ 0 04 μg/ml (C V $=4\ 2\%$), and the 5 0 μg/ml QC showed a mean of 5 02 $\pm$ 0.16 μg/ml (C V $=3\ 3\%$)

Canine bolus study

Chromatograms showing blank canine plasma, plasma from a human kidney allograft patient not receiving MZB, a 2 5 μg/ml MZB calibration standard,

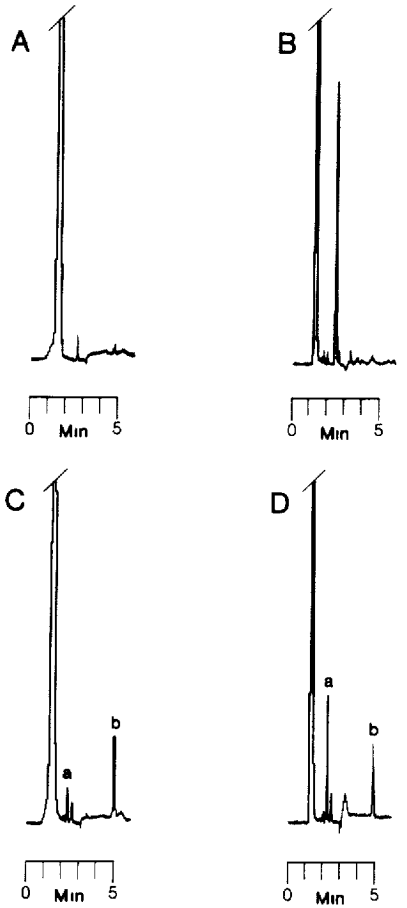

Fig 1 Chromatograms of (A) blank canine plasma, (B) human kidney transplant patient plasma not receiving mizoribine, (C) 2 5 μg mizoribine per ml plasma standard, and (D) canine plasma 0 75 h after receiving a 5 mg/kg intravenous bolus of mizoribine Peaks a = mizoribine, b = internal standard

and a canine sample containing 9 76 μg/ml MZB are shown in Fig 1A–D, respectively There were no interfering substances present in canine or human plasma The retention time of MZB was 2 36 min and the I S eluted at 5 04 min

DISCUSSION

The successful use of MZB for immunosuppression of renal allograft recipients in Japan is well documented [2,6,9,10] In the United States, Gregory et al [11] have demonstrated synergistic effects of orally administered MZB and

cyclosporine in prolonging canine renal allograft survival Currently, MZB is undergoing investigation in a canine renal allograft model of local immunosuppression at our center [12] However, due to the limited success in developing a standardized HPLC procedure for quantifying MZB levels in plasma, the majority of studies have relied on the analytical services of the manufacturer to obtain plasma levels The difficulty in analyzing MZB is due primarily to the hydrophilic nature of the drug and its very poor solubility in most organic solvents MZB has been retained using amine columns but the chromatographic conditions precluded the use of an appropriate I.S

We present here a reversed-phase HPLC method using octanesulfonic acid as a modifier that successfully quantified 0 25 μg/ml MZB in plasma using an ODS column The procedure is an improvement over previous techniques because it utilizes an I S as well as a column packing and mobile phase commonly used in reversed-phase HPLC The method shows excellent linearity, precision, and accuracy in the range 0 25–10 μg/ml. Under these conditions, there were no interfering components in either canine or human kidney allograft plasma samples The sample procedure requires precipitation of plasma protein and a clean-up extraction with dichloromethane Prior to injection, the samples were neutralized and the sample compartment was covered with aluminum foil since MZB decomposes in an acid medium when exposed to light The pH of the mobile phase showed little effect in changing the retention of MZB, but was useful in moving adjacent plasma components As the pH of the mobile phase was increased to 6 5 or greater, certain plasma substances coeluted with the I S Due to the pH and methanol content of the mobile phase, a daily column wash with 80% methanol extended the life of the column to accommodate >500 injections The very small injection volume also contributed to column stability

As approvals for MZB use in experimental and clinical organ transplantation increase [13], so will the need for reproducible, sensitive, and simple analytic methods for MZB quantitation The HPLC method reported here is currently being used to monitor plasma MZB levels in canine renal allograft recipients receiving drug intrarenally via an implantable programmable infusion pump system [14] An evaluation of human kidney allograft plasma also suggests that the method described herein is applicable to human pharmacokinetic studies

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