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Determination of Moxalactam in Human Body Fluids by Liquid Chromatographic and Microbiological Methods

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High-performance liquid chromatographic methods for determination of the isomers of moxalactam in plasma and urine have been developed. Conventional reverse-phase chromatography was used for plasma assays, and an ion-pairing reagent was included for urine assays. Detection limits were 1.5 μg/ml of plasma and 7.5 μg/ml of urine. The high-performance liquid chromatographic assays were extensively compared with a microbiological assay (detection limit, 1 μg/ml), using samples from human volunteers to whom moxalactam had been administered as well as plasma and urine from untreated humans, to which moxalactam was added. The correlations between the assays were quite good, but the precision and accuracy of the high-performance liquid chromatographic methods were superior. Both types of assays were used in a study of the stability of moxalactam-containing samples at various temperatures.

Moxalactam {LY 127935, 7β-[*RS*-[carboxy(4-hydroxyphenyl)acetyl]amino]-7α-methoxy-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-8-oxo-5-oxa-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, disodium salt} is a new semisynthetic β-lactam antibiotic. It consists of the *R*- and *S*-epimers in about a 1:1 ratio. Moxalactam has displayed activity against a wide spectrum of microorganisms in vitro (1, 8, 14), and early clinical evaluations appear promising (3). Brief reports of liquid chromatographic and microbiological determinations of moxalactam have appeared recently (10, 13). Antibiotics have traditionally been determined by microbiological methods, although a variety of approaches have been proffered. Liquid chromatographic methods offer molecular specificity and as a result are being used more frequently for assay purposes (7, 11, 12). This paper describes a high-performance liquid chromatographic (HPLC) method for the determination of moxalactam in human body fluids and compares the results obtained with those acquired by using a conventional microbiological method. These methods have been used to study the stability of moxalactam under various storage conditions and have been successfully applied to plasma and urine samples obtained from human volunteer participants in a pharmacokinetic study.

MATERIALS AND METHODS

Reference standard. Reference standard moxalactam was stored at −20°C in sealed ampoules. After an ampoule was opened, the material was held overnight in a desiccator containing a saturated solution of potassium carbonate. Karl Fischer moisture determinations were then performed on a portion of the standard, and the remainder was weighed out immediately into volumetric flasks. In subsequent calculations, potency was adjusted for moisture uptake. Samples to be used at a later date were stored desiccated at −20°C. This procedure obviates the need for daily weighings and moisture determinations. Moxalactam is furnished as a disodium salt; however, all concentrations listed in this paper refer to the free diacid. Standard solutions (1 mg/ml) were prepared every other day by reconstitution of an appropriate quantity in 0.1 M ammonium acetate (pH 6.8) and then stored at 4°C.

HPLC determination in plasma. A standard curve was prepared in duplicate by spiking human serum (Grand Island Biological Co.) to yield samples containing moxalactam at concentrations of 5, 10, 25, and 50 μg/ml. The spiked serum samples together with the plasma or serum samples to be assayed were then processed simultaneously in groups of 12 or less. Five hundred microliters of serum or plasma was transferred to a 1.5-ml polypropylene tube, and 500 μl of ice-cold methanol was added. The contents of the tube were mixed and twice centrifuged (Eppendorf no. 5412) for 5 min at 12,800 × *g* and 0°C. Five hundred microliters of the supernatant was mixed with 500 μl of ice-cold 0.1 M citrate (pH 6.5), and the mixture was placed in a freezer until it was chromatographed. Standards should be chromatographed alternately with samples and used to construct a calibration curve of peak height versus concentration. Standards prepared with serum or plasma give identical peak heights. Cerebrospinal fluid samples were prepared by mixing 67 μl of cerebrospinal fluid with 200 μl of

methanol–0.1 M citrate (1:2), pH 6.5, omitting centrifugation.

The chromatographic system consisted of a pump (6000A, Waters Associates), an automatic sample processor (WISP 710A, Waters Associates), a column (30 by 4.6 mm; Chromegabond C18, ES Industries) equipped with a guard column (guard column [Waters Associates] packed with CO:PELL ODS [Whatman]), and a multiwavelength ultraviolet detector (model 770, Waters). The detector wavelength was set at 270 nm, and the sensitivity was set at 0.02 absorbance units full scale (AUFS). The degassed mobile phase, consisting of 0.1 M ammonium acetate (95%)–acetonitrile (5%), pH 6.5, was pumped at 1.5 ml/min. The injection volume was 70 μl, and samples were injected at 10-min intervals.

HPLC determination in urine. The chromatographic apparatus used for urine assays was similar to that used for plasma assays, except for substitution of a DuPont column (25 cm by 4.6 mm, 6-μm Zorbax TMS). The detector wavelength was set at 280 nm to enhance selectivity, and the sensitivity was set at 0.1 AUFS. The mobile phase consisted of 5 mM *n*-heptylamine in 11% methanol–distilled water, adjusted to pH 6.0 with concentrated phosphoric acid. The mixture was filtered (0.22-μm type GS, Millipore Corp.) and degassed by sonicating it for 2 min with vacuum applied. The flow rate was 1.4 ml/min; the injection volume was 50 μl. Retention times on a single column decreased over several days. This could be compensated for by lowering the percentage of methanol in the mobile phase. Retention is highly dependent on methanol content, and the amount required may vary from column to column.

Urine samples, prepared by mixing 1 volume of urine with 4 volumes of mobile phase, were refrigerated until they were chromatographed. A standard curve was prepared by mixing 1.0 ml of urine and an appropriate volume of standard solution and diluting the mixture to 5.0 ml with mobile phase. Curve points equivalent to 40, 80, 140, 240, and 400 μg/ml of urine were used.

Microbiological assays. A standard agar diffusion procedure (4), using petri dishes measuring 100 by 20 mm, was used for microbiological assays. To each dish was added 10 ml of melted and cooled (50°C) agar medium no. 1 seeded with 0.5% of an overnight broth culture of *Escherichia coli* ATCC 10536. After the agar hardened, four 6-mm wells were cut in each agar plate. Thirty-microliter samples of standard moxalactam solutions (1, 2.5, 5.0, and 10 μg/ml) were added to the wells of each of nine plates. For each sample of plasma, serum, urine, or cerebrospinal fluid, 30-μl samples, were placed in two wells on each of three plates. Similar volumes of reference standard maxalactam (5 μg/ml) were added to the remaining wells of these plates. Plates so prepared were incubated at 30°C for 16 to 18 h, at which time zones of inhibition were measured to the nearest 0.1 mm on a Lilly-Fisher zone reader. The means of the zones of inhibition (in millimeters) were plotted against logs of standard concentrations to produce the standard curve. Serum or plasma samples were assayed against standards prepared in serum or plasma. Urine and cerebrospinal fluid samples were assayed against standards prepared with 0.1 M phosphate buffer, pH 6.0.

Characterization of HPLC and microbiological determinations. The precision and accuracy of the HPLC and microbiological procedures when applied to plasma were assessed on five samples of spiked plasma, five determinations on each sample being made on each of 3 or 4 days. The plasma samples used were prepared simultaneously and stored at −20°C until analyzed. Concentrations obtained after day 1 were corrected for decomposition, using rate constants obtained as described below.

Plasma and urine samples obtained from participants in a pharmacokinetic study were assayed by both HPLC and microbiological methods. These participants, eight adult male volunteers, had received 500 mg of moxalactam twice intramuscularly and once intravenously on separate days. Twelve plasma samples were drawn at appropriate times during an 8-h interval after dosing, and urine samples were collected at 2, 4, 6, 8, 12, and 24 h.

Stability of moxalactam in body fluids. Fresh plasma, serum, cerebrospinal fluid, and urine samples were obtained from nonmedicated human volunteers and were spiked with moxalactam to yield 15 and 50 μg/ml of plasma, 30 μg/ml of serum, 10 μg/ml of cerebrospinal fluid, and 100 and 500 μg/ml of urine. Aliquots were immediately dispensed into individual tubes, so that sampling would not be necessary at the time of analysis. The tubes were divided and refrigerated (+4°C), frozen (−20 or −72°C), or held at room temperature. Samples were removed at intervals consistent with storage conditions and analyzed in triplicate by the microbiological and HPLC procedures.

The rate of degradation of moxalactam was estimated by assuming that it was a first-order process. A conventional linear least-squares fit was made to the natural logs of concentration-versus-time data. The slope of this line is the first-order rate constant (k). The stability of moxalactam at −72°C was followed for 70 days, which was insufficient (given the variability of the assays) to precisely determine rate constants. Results from both determinations were used individually to generate rate constants. An average of the rate constants, each one weighted in proportion to the standard error of the other, was used to calculate the times required for 5% decomposition (t_{95} values).

RESULTS

Chromatograms typical of the HPLC determination of moxalactam in plasma are depicted in Fig. 1. The resolution of the *R*- and *S*-epimers from each other and from plasma constituents was evident. Cerebrospinal fluid samples gave chromatograms cleaner than those from plasma.

Because of the relative complexity of urine, more selectivity was necessary in order to chromatographically determine moxalactam in urine. Addition of *n*-heptylamine to the mobile phase provided special selectivity for moxalactam, since moxalactam contains two carboxylic acid groups. A reverse-phase column more resistant to attack by ion-pairing reagents, such as *n*-heptylamine, was used. Typical chromatograms are depicted in Fig. 2. The chromatogram of spiked urine (Fig. 2B) is also representative of

chromatograms or urines from volunteers receiving moxalactam. As with the plasma determination, the resolution of the *R*- and *S*-epimers from each other and from endogenous substances was clear. Other drugs were not tested for potential interference.

The precision and accuracy of the HPLC and microbiological plasma assays are summarized in Table 1. The HPLC determination exhibited a negative mean bias of 3%, with an overall relative standard deviation of from between 3 and 5% at higher concentrations to 10% at concentrations below 7 μg/ml. The precision of determination of the individual isomers (not listed in Table 1) was somewhat better than the precision for the sum of the two. For example, at 18 and 40 μg/ml, the relative standard deviations for the *R*-epimer were 4.2 and 2.8%, respectively, and the relative standard deviations for the *S*-epimer were 5.2 and 3.2%. The microbiological procedure showed an 8% negative mean bias, with an overall relative standard deviation of 11%. Between- and within-day variations contributed approximately equally to the total variabilities of both plasma methods. Table 2 lists the results of a single-day characterization of the urine assays. Over the range 50 to 400 μg of moxalactam per ml of urine, the relative standard deviations were 3 and 4% for the HPLC and microbiological methods, respectively. The relative biases of the assays on urine were smaller than those on plasma.

The two procedures were successfully applied

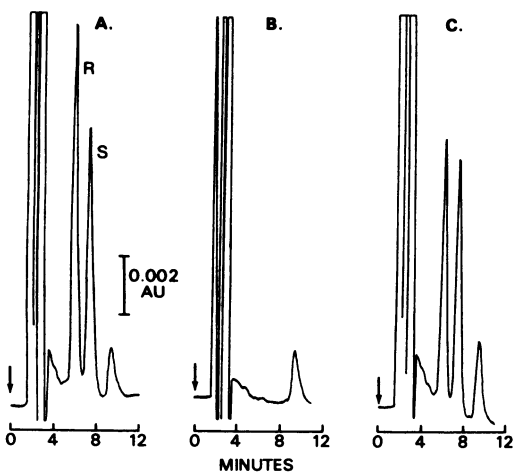

FIG. 1. *Liquid chromatograms of plasma extracts. (A) Plasma spiked with 50 μg of moxalactam per ml (peaks for the R- and S-epimers are marked). (B) Predose patient's plasma. (C) Sample at 45 min from patient dosed intravenously with 500 mg moxalactam. AU, Absorbance unit.*

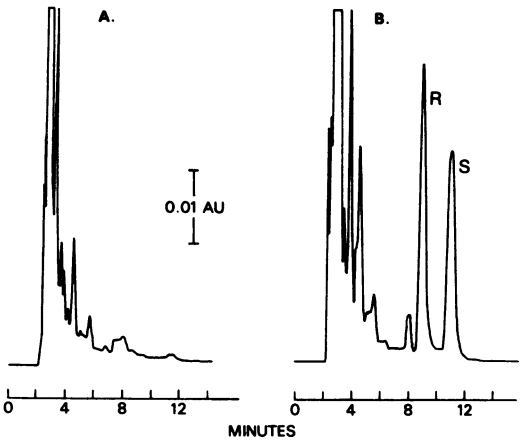

FIG. 2. *Liquid chromatograms of diluted urines. (A) Blank urine. (B) Urine spiked with 300 μg of moxalactam per ml (peaks for the R- and S-epimers are marked). AU, Absorbance unit.*

to samples of plasma and urine obtained from human volunteer participants in a pharmacokinetic study. Agreement between the two methods was generally quite good. Typical curves after intramuscular administration of moxalactam are shown in Fig. 3. Statistical analysis of the data for samples on which the two determinations were performed within a few days of each other gave a correlation coefficient of 0.93 (n = 120). The regression equation was as follows: (microbiological) = (0.92) (HPLC) + 0.51 μg/ml. The lower results of microbiological determination were consistent with the bias observed with plasma samples to which moxalactam was added (Table 1). All microbiological assays of urine were performed upon receipt of samples; some samples were subsequently assayed by HPLC. Regression analysis of the data gave a correlation coefficient of 0.98 (n = 27) and the following equation: (microbiological) = (1.03) (HPLC) + 9.7 μg/ml.

Two typical sets of stability results are fit to first-order decay kinetics in Fig. 4. Correlation coefficients from plots like those in Fig. 4 were >0.77 for all data, and most were >0.94. The agreement between rate constants determined microbiologically and by HPLC was within experimental error in most cases. The influence of moxalactam concentration on first-order rate constants is shown in Table 3. Rates of decomposition in both plasma and urine containing different concentrations of moxalactam were very similar, lending credence to the supposition of first-order behavior.

In a separate experiment, two samples of urine spiked with moxalactam were adjusted to pH

TABLE 1. *Precision and accuracy of determinations of moxalactam in plasma*

Moxalactam concn (μg/ml of spiked plasma)	Determination method									
	Microbiological (n = 15)					HPLC (n = 20)				
	Mean moxalactam concn (μg/ml)	Relative bias (%)	Relative standard deviation (%)			Mean moxalactam concn (μg/ml)	Relative bias (%)	Relative standard deviation (%)		
			Between-day	Within-day	Total			Between-day	Within-day	Total
3.0	2.9	−3.0	3.5	9.0	9.7	3.0	0	5.2	13.7	14.6
7.0	5.9	−15.0	10.9	6.8	12.9	6.6	−6.0		9.0	9.0
18.0	18.0	−0.1		9.5	9.5	17.2	−4.6	2.8	3.6	4.6
40.0	35.8	−10.7	6.7	7.2	9.8	38.8	−3.1	3.6	3.0	4.7
90.0	78.6	−12.6	4.1	12.0	12.7	89.1	−1.0	2.5	1.1	2.7

TABLE 2. *Precision and accuracy of determinations of moxalactam in urine*

Moxalactam concn (μg/ml) in spiked urine	Determination method (n = 5)					
	Microbiological			HPLC		
	Mean moxalactam concn (μg/ml)	Relative bias (%)	Relative standard deviation (%)	Mean moxalactam concn (μg/ml)	Relative bias (%)	Relative standard deviation (%)
50	48.3	−3.4	5.3	52.2	+4.5	5.3
150	145	−3.1	3.9	151	+0.7	1.3
400	382	−4.4	2.7	395	−1.2	1.2

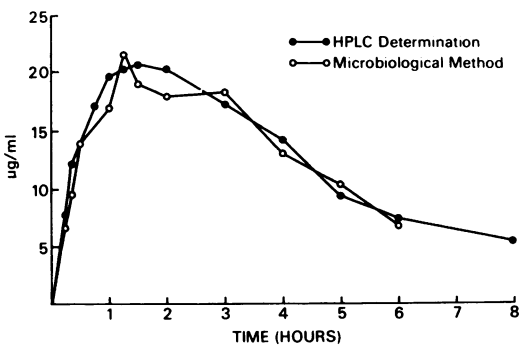

FIG. 3. *Plasma concentrations of moxalactam after a 500-mg intramuscular dose.*

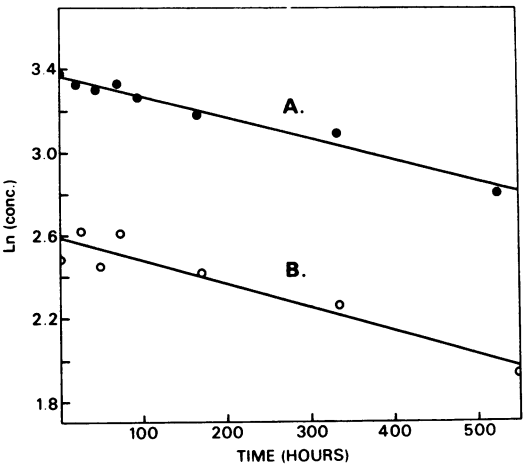

FIG. 4. *Decomposition of refrigerated (4°C) moxalactam samples. (A) Serum monitored by HPLC. (B) Plasma monitored microbiologically.*

values of 4.0 and 7.0 and stored at room temperature. These urines were monitored by HPLC with the following results: k (pH 4) = 0.071 ± 0.017 day^{-1} and k (pH 7) = 0.102 ± 0.017 day^{-1}. All of the stability results are summarized in Table 4.

Since it was possible that the isomers of moxalactam interconverted in stored samples, rate constants were also calculated (from HPLC data) for the R-isomer alone and compared with overall (for both epimers) rates. Although rate constants for the R-isomer alone appeared to be higher in some cases, the differences were not statistically significant. In a separate experiment, no differences in moxalactam levels were detected between three sets of samples of plasma, serum, and cerebrospinal fluid that were frozen and thawed and an identical set of samples which had not been frozen.

DISCUSSION

Comparison of determinations. The two approaches to measurement of moxalactam in

TABLE 3. *Influence of moxalactam concentration on measured rate of decomposition*

Temp (°C)	Measured rate of decomposition per day in:			
	Plasma containing moxalactam at:		Urine containing moxalactam at:	
	15 μg/ml	50 μg/ml	100 μg/ml	500 μg/ml
+25	0.52	0.50	0.089	0.089
+4	0.026	0.025	0.0045	0.0045
−20	0.0047	0.0049	ND[a]	ND

[a] ND, Not determined.

TABLE 4. *Stability of moxalactam in biological media*

Temp (°C)	t_{95}			
	Serum	Plasma	Cerebrospinal fluid	Urine
+25	ND[a]	2.4 h	2.5 h	13.9 h
+4	49 h	50 h	37 h	11 days
−20	9 days	11 days	5.5 days	>30 days
−72	>30 days	>30 days	>30 days	ND

[a] ND, Not determined.

plasma and urine may be compared on the basis of several factors. Absolute detection limits are similar for plasma (1.5 and 1.0 μg of moxalactam per ml of plasma by HPLC and bioassay, respectively), but are different for urine (5 to 10 μg/ml by HPLC, 1.0 μg/ml by bioassay). Since the preparation procedures for HPLC determinations consist of dilutions, they can be performed with any convenient volume so long as there is sufficient final volume for chromatographic injection. Thus, HPLC may be performed with as little as 50 μl of plasma or 25 μl of urine, whereas the microbiological procedure requires 180 μl (less for concentrations above 10 μg/ml). Due to increasing contributions from detector noise at lower concentrations, the precision of the HPLC plasma method is a function of concentration. The overall standard deviation of the bioassay procedure applied to plasma (11%) is apparently not a function of concentration (Table 1) and is consistent with other assays of this type. Except near its detection limit, the precision of the HPLC determination is superior.

The negative biases exhibited by both methods with plasma are of concern, but the magnitude in either case was less than the overall deviation of the methods. Proper sample handling during the assay is an important consideration, due to the instability of moxalactam in biological media. Samples were kept cold during preparation, and handling time was minimized. During collection of the statistical data in Table 1, standards were chromatographed before samples of unknowns. Subsequently, it was found that chromatographing standards both before and after chromatographing unknowns might eliminate the negative bias of the HPLC determination.

In summary, the obvious difference between the methods is in selectivity: the HPLC methods measure chemical entities, including the two isomers of moxalactam, whereas the microbiological assays measure total antimicrobial activity. Both approaches are potentially subject to interferences. The HPLC methods have advantages with respect to precision, accuracy, sample volume requirement, and speed (overnight incubation is not required). The microbiological procedures are preferred in terms of sensitivity (minimal quantity that can be detected) and sample throughput (many samples may be incubated simultaneously). These conclusions are consistent with those from similar comparisons of these procedures as applied to other antibiotics (2, 5, 6, 9).

Stability of moxalactam samples. The stability of a drug under various conditions can have important implications for sample handling. Both acids and bases catalyze interconversion of the isomers of moxalactam and degradation to several products (A. D. Kossoy, unpublished data). The influences of medium and temperature on the stability of moxalactam are reported in Table 4. The effect of temperature is clear; only at −72°C do moxalactam samples exhibit long-term stability. Moxalactam behaves similarly in serum and plasma samples. It is less stable in cerebrospinal fluid, particularly at −20°C, and is more stable in urine samples.

These data on stability have clear implications for sample handling. Losses during handling are additive, each phase contributing to error. To obtain 5% overall accuracy, for example, the analyst should be guided by t_{98} or t_{99} values instead of the t_{95} values listed in Table 4. The t_{98} value may be obtained by multiplying t_{95} by 0.39, and t_{99} may be obtained by multiplying t_{95} by 0.20. The ideal procedure would involve immediate analysis of collected samples, but when this is not possible, immediate storage at −70°C is recommended.

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