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Note

Modified method for the determination of moxalactam in plasma by high-performance liquid chromatography

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Moxalactam is a semisynthetic β-lactam antibiotic in which the sulphur of the cephem ring has been replaced by oxygen. It has an extended antibiotic spectrum including such organisms as *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Bacteroides fragilis*, as well as the *Enterobacteriaceae* and Gram-positive cocci [1, 2]. A method for measuring moxalactam in plasma and tissue was required in order that a study of the effects of the drug following abdominal surgery could be carried out.

Although a number of methods for measuring moxalactam in biological material exist [3—8] only three were found which used an internal standard [6—8], a feature considered desirable for ease of quantitation. The method of Ziemniak et al. [8] was selected for further investigation because it showed the best separation of moxalactam isomers, and because all reagents were readily available to us.

In our hands, this method [8] did not produce satisfactory results, primarily because the recovery of the internal standard, allopurinol, was near zero and that of the moxalactam was unacceptably small. A number of modifications have been made.

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EXPERIMENTAL

Apparatus

A Waters high-performance liquid chromatographic (HPLC) system was used (Waters Assoc., Milford, MA, U.S.A.). This consisted of an M6000 pump, M440 UV absorbance detector fitted with a 280-nm filter and a WISP 710 sample processor. The detector output was processed using a Spectra-Physics 4100 computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.) which had been programmed in this laboratory to respond to the chart mark contact closure on the WISP, allowing unattended operation.

A Waters C_{18} μBondapak, or a Serva, octadecyl 5-μm reversed-phase column (Serva Feinbiochemica, Heidelberg, F.R.G.) was used for separation of the moxalactam isomers and the internal standard.

Reagents

Tromethamine (Tris) was buffer grade (Strem Fine Chemicals, Farmington, MO, U.S.A.). Acetonitrile was Baker HPLC grade, and all other reagents were Baker Analyzed Reagents (Baker, Phillipsburg, NJ, U.S.A.), and were used without further purification.

Chromatography solvent was prepared by adding 38 ml of acetonitrile to 1 l of 0.05 mol/l ammonium acetate solution which had been buffered to pH 5.5 with acetic acid– water (10:90). The solvent was filtered and degassed by passage through a 0.5-μm filter (Millipore FHUP 047 00). It was necessary to wet the hydrophobic filter disk with 0.5 ml of methanol before adding the aqueous solvent.

Standards

Allopurinol (internal standard) was prepared by crushing a 300-mg Zyloprim tablet (Wellcome, Auckland, New Zealand) and dissolving the drug in 20 ml of a 0.1 mol/l sodium hydroxide solution using an ultrasonic bath. The solution was made up to 100 ml with water and filtered. When used with plasma standards containing more than 10 μg/ml moxalactam, this solution was diluted approximately 1:70 with water. For plasma standards containing less than 10 μg/ml, the stock was diluted 1:350 with water. The exact concentration is not critical since the same volume is used for all samples within a batch.

Moxalactam diammonium was provided by Lilly (Papatoetoe, New Zealand). An aqueous stock solution containing 2.5 mg/ml moxalactam was prepared by dissolving 65.4 mg of the salt in water and diluting to 25 ml. Plasma standards over the range 5—100 μg/ml were prepared by dilution of the stock solution with plasma. Aliquots of plasma standards were stored at —70° C until required. These standards were used to measure recoveries from each run. An aqueous calibration standard containing moxalactam diammonium was prepared in water. Aliquots containing 0.5 ml of this standard were frozen at —70° C until required.

Procedure

All glassware was silanized by leaving overnight in an enclosed container saturated with dimethyldichlorosilane vapour.

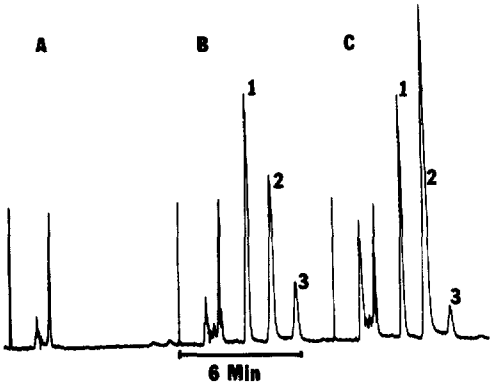

Fig. 1. Chromatograms showing a plasma blank (A), a specimen from a patient with a measured concentration of combined moxalactam isomers of 72 μg/ml (B) and a plasma standard containing 100 μg/ml moxalactam (C). Peaks: 1 = allopurinol, internal standard; 2 and 3 = D- and L-isomers of moxalactam, respectively.

A 500-μl aliquot of sample or plasma standard was placed in a 10-ml conical centrifuge tube together with 500 μl of water and 6 ml of ethyl acetate. The mixture was vortexed for 10 s. Then 50 μl of concentrated hydrochloric acid were added and vortexing was continued until a gel formed throughout the whole mixture. The tubes were centrifuged at 1100 *g* for 5 min and 5 ml of the supernatant transferred to another conical centrifuge tube containing 1 ml of 0.05 mol/l (6.06 g/l) Tris—HCl buffer at pH 8.0. The mixture was vortexed for 30 s and then centrifuged for 5 min. The lower aqueous layer was allowed to clear of any cloudiness by standing the tubes on the bench for 5—10 min. The upper solvent layer was carefully removed and discarded. A 200-μl volume of the aqueous phase was mixed with 200 μl of internal standard and 50 μl were injected into the HPLC system. The mobile phase flow-rate was 2.5 ml/min. Chromatographic traces achieved using the method are shown in Fig. 1. Specimens found to contain less than 10 μg/ml moxalactam were reprocessed using appropriate plasma standards and the dilute internal standard solution at an increased detector sensitivity. Specimens containing more than 100 μg/ml were diluted to bring the moxalactam concentration below 100 μg/ml and reprocessed.

Standardization

Calibration standard (200 μl) was mixed with 200 μl of internal standard and 50 μl of the mixture were injected into the HPLC system. The areas of the peaks for the two moxalactam isomers were measured and concentrations proportional to their areas assigned so that the total concentration was 50 μg/ml. The integrator was then calibrated to obtain response factors for these concentrations relative to the internal standard. According to Konaka et al. [7] the two moxalactam isomers show the same UV characteristics.

Plasma standards, which had been extracted, were chromatographed and recoveries measured. These results were used to calculate the moxalactam concentration in specimens from patients. A standard curve over the range 0—100 μg/ml was found to be linear with a regression slope of 0.7762, an *y*-intercept of −0.49 and a standard deviation about the line of 0.52.

While attempting to follow the method of Ziemniak et al. [8] two major problems were encountered. Firstly, in our hands, the recovery of the internal standard at the extraction stage was very low and, secondly, the 73—81% recovery of moxalactam isomers reported by Ziemniak et al. [8] could not be achieved, 57—65% being recovered. In an effort to improve the recovery of moxalactam, the following procedural changes were investigated. An equal volume of water was added to measured plasma samples and standards before extraction, decreasing amounts of hydrochloric acid were added, the quantity of ethyl acetate used to extract the drug was doubled and this ethyl acetate was added to the plasma before the hydrochloric acid. The octanol wash as used by Ziemniak et al. [8] was found to give no advantage and was not used.

RESULTS AND DISCUSSION

None of the procedures tested satisfactorily recovered allopurinol and it was reluctantly decided to add the internal standard to the final extract immediately prior to injection.

When 500 μl of water were added to 500 μl of plasma prior to acidification and extraction, a recovery for the total moxalactam isomers of 71.5 ± 2.1% was obtained compared with 65.7 ± 1.46% in the absence of additional water.

When progressively smaller quantities of hydrochloric acid were added, the recoveries of moxalactam shown in Fig. 2 were obtained. Using smaller quantities of hydrochloric acid resulted in a marked reduction in protein precipitation and a slightly improved moxalactam recovery with a progressive change in the ratio of the two isomers (Fig. 3). It is tempting to ascribe losses on total protein precipitation, as found when 200 μl of hydrochloric acid were used, to entrapment of the drug molecules within the protein flocculate. Failure to use acid in the extraction mixture resulted in a total lack of recovery

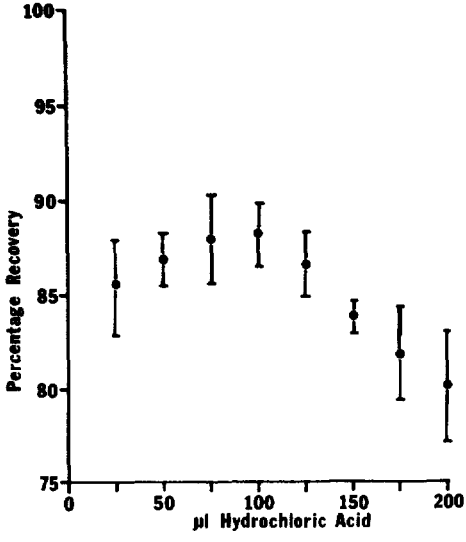

Fig. 2. Percentage recovery of moxalactam isomers using different quantities of concentrated hydrochloric acid.

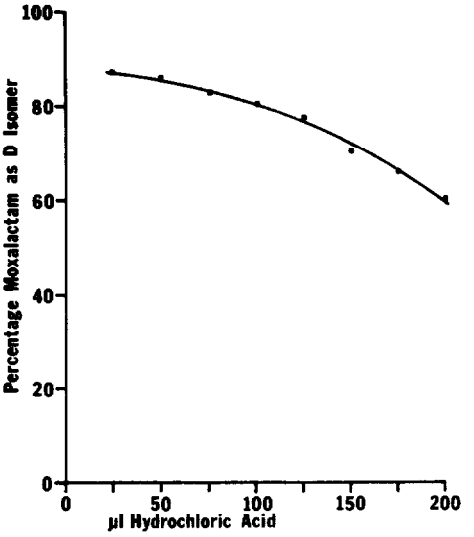

Fig. 3. Change in the quantity of the D-isomer of moxalactam as a percentage of the total recovered when extractions were carried out in the presence of different amounts of concentrated hydrochloric acid.

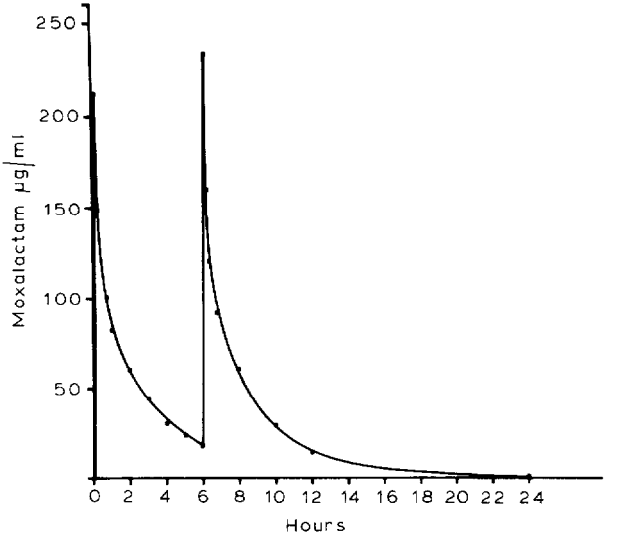

Fig. 4. Changes in plasma concentrations resulting from the intravenous administration of two 1-g doses of moxalactam 6 h apart.

of moxalactam. Recoveries using volumes of acid less than 25 µl were not examined. Because of the combination of these effects, 50 µl of hydrochloric acid were selected for routine use.

During the initial examination of the procedure, doubling the quantity of ethyl acetate used for extraction to 6 ml was found to yield recoveries of moxalactam which were 11% higher than those obtained when the 3 ml recommended by Ziemniak et al. [8] were used.

Ziemniak et al. [8] reported no interference from a number of drugs likely

to be isolated from specimens collected from critically ill patients and no interfering substances were detected by us when analysing specimens from the trial for which this method was established.

As a result of these examinations, the procedure described above has been adopted. In our hands, recoveries of 79 ± 1.72% for moxalactam have been achieved within runs, giving a coefficient of variation of 2.19% at a nominal concentration of 100 μg/ml. The between-run variation is somewhat greater necessitating the use of plasma standards with each batch. Over a period of four months, the method gave a mean recovery of 78.3 ± 6.3% and a coefficient of variation of 8.0%. The method has now been used, in conjunction with a trial, to measure moxalactam levels in the plasma from patients undergoing abdominal surgery whose plasma concentrations vary between 450 and 10 μg/ml over a 6-h period. A plot of levels versus time for one patient given two 1-g doses of moxalactam 6 h apart is shown in Fig. 4. Full details will be published elsewhere.

CONCLUSION

A modified method for the measurement of moxalactam in plasma is described. A number of problems encountered with the original method have been investigated and eliminated or reduced. The method is rapid and sufficiently reproducible for clinical use.

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