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DETERMINATION OF A NEW ANTIBACTERIAL AGENT (AT-2266) AND ITS METABOLITES IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method has been developed which enables accurate determination of a new synthetic antibacterial agent, AT-2266, and its metabolite, M-2, in plasma, and AT-2266 and its five metabolites, M-1, M-2, M-3, M-4 and M-5, in urine. AT-2266 is extracted as ethyl carbamate with chloroform containing 1% ethyl chloroformate and assayed on a liquid chromatograph equipped with an ultraviolet detector at 340 nm.

Accurate determinations are possible over a concentration range of 0.1—10 μg/ml AT-2266 in plasma, and 1—500 μg/ml AT-2266 in urine. The coefficient of variation at the 2 μg/ml level of AT-2266 is 1.9% (n = 6). The minimum detectable concentrations of AT-2266 in plasma and urine are 0.01 μg/ml and 0.1 μg/ml, respectively, and those of other metabolites are similar to those of AT-2266. Plasma levels and urinary excretion of AT-2266 in a man following single oral administration (400 mg) have also been determined.

INTRODUCTION

The compound 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-1,8-naphthyridine-3-carboxylic acid, AT-2266 ([1], Fig. 1), is a new potent synthetic antibacterial agent.

After oral dosing of AT-2266 in humans the unchanged drug and a metabolite, M-2 (Fig. 1), were detected in plasma, and four other metabolites, M-1, M-3, M-4 and M-5, were also detected in minute quantities together with the unchanged drug and M-2 in urine [2]. In order to study the pharmacokinetics of AT-2266, simple and selective assay methods for unchanged drug and its metabolites are necessary.

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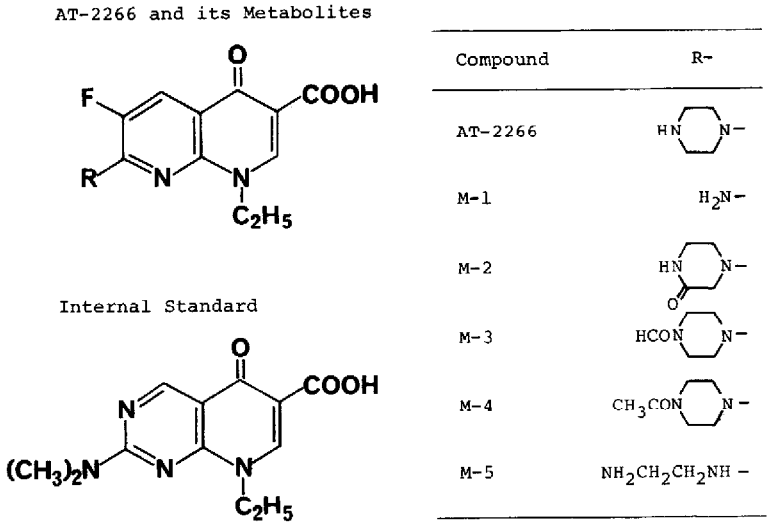

Fig. 1. Chemical structures of AT-2266, its metabolites and the internal standard.

This paper describes a high-performance liquid chromatographic (HPLC) method with a good reproducibility and specificity for determining AT-2266 and its metabolites in plasma and urine.

EXPERIMENTAL

Chemicals and reagents

AT-2266, M-1 (7-amino-1-ethyl-6-fluoro-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid), M-2 [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(3-oxo-1-piperazinyl)-1,8-naphthyridine-3-carboxylic acid], M-3 [1-ethyl-6-fluoro-7-(4-formyl-1-piperazinyl)-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid], M-4 [7-(4-acetyl-1-piperazinyl)-1-ethyl-6-fluoro-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid] and M-5 [7-(2-aminoethylamino)-1-ethyl-6-fluoro-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid] were synthesized in our laboratories [1]. 8-Ethyl-5,8-dihydro-2-dimethylamino-5-oxopyrido(2,3-*d*)-pyrimidine-6-carboxylic acid was used as the internal standard [3]. The chemical structures of these compounds are shown in Fig. 1. Ethyl chloroformate was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals used were of analytical-reagent grade.

Standard solutions

The compounds were dissolved in methanol (50—100 μg/ml) in a 50—60°C water bath, and diluted with methanol. The internal standard was dissolved in the minimum volume of 0.1 *M* sodium hydroxide and diluted with 0.2 *M* phosphate buffer (pH 7.4) to concentrations of 1 μg/ml for plasma and 20 μg/ml for urine. These standard solutions were stored in a refrigerator (about 4°C).

Instrumentation

HPLC was carried out using a Waters Model ALC/GPC 204 liquid chromatograph equipped with a dual-delivery pump (Model 6000A), an automatic sampler (Model 710B), an ultraviolet (UV) detector (Model 440) with a 340-nm-wavelength filter and a recorder with integration (Data Module, Model 730). The stainless-steel column (30 cm × 4 mm I.D.) packed with μBondapak C_{18} (particle size 10 μm) was used (Waters Assoc.). The following three mobile phases were used at a flow-rate of 1.5 ml/min: (1) methanol—0.1 *M* citric acid—acetonitrile (9:5:1), (2) methanol—0.1 *M* citric acid—acetonitrile (6:5:1), (3) methanol—5% acetic acid—acetonitrile (6:10:1).

Assay procedure for AT-2266 and M-2 in plasma

To 1 ml of plasma sample were added 1 ml of the internal standard solution (1 μg) and 3 ml of chloroform containing 1% of ethyl chloroformate in a glass-stoppered 15-ml centrifuge tube. The tube was shaken for 10 min and centrifuged for 10 min at 2000 *g*. The organic layer (2 ml) was transferred to another tube and evaporated to dryness under a gentle stream of air at 50°C. The residue was dissolved in 200 μl of methanol, and a 20-μl aliquot of the solution was injected into the liquid chromatograph with mobile phase 1.

Assay procedure for AT-2266 and its metabolites in urine

AT-2266, M-2 and M-5 (procedure U-1). To 1 ml of urine sample were added 1 ml of the internal standard solution (20 μg) and 3 ml of chloroform containing 1% ethyl chloroformate in a glass-stoppered 15-ml centrifuge tube. The tube was shaken and centrifuged. The organic layer (2 ml) was evaporated to dryness. The residue was dissolved in 2 ml of methanol, and a 30-μl aliquot of the solution was injected into the liquid chromatograph with mobile phase 2.

M-1, M-3 and M-4 (procedure U-2). To 1 ml of urine sample were added 1 ml of the internal standard solution (20 μg) and 3 ml of chloroform in a glass-stoppered 15-ml centrifuge tube. The tube was treated as described in the above procedure U-1, and the liquid chromatograph was used with mobile phase 3.

Calibration curves

The drug solutions, used in the construction of the calibration curve, were prepared by diluting the stock solution with methanol, pipetted into glass-stoppered 15-ml centrifuge tubes and evaporated to dryness. The amounts used for plasma calibration curve were in the range of 0.1—10 μg/ml AT-2266 and 0.03—2.5 μg/ml M-2. The tubes were treated according to the assay procedure. Peak-height ratios of AT-2266 and M-2 to the internal standard were measured and plotted against the amount of AT-2266 and M-2 seeded.

The amounts used for urine calibration curve (procedure U-1) were in the range of 1—500 μg/ml AT-2266, 1—125 μg/ml M-2 and 1—20 μg/ml M-5. Other minor metabolites, M-1, M-3 and M-4, were added in the same range 1—20 μg/ml (procedure U-2).

Stability of AT-2266 and its metabolites

The stability of AT-2266 and its metabolites in methanol, plasma and urine was examined. The methanol solutions (50 μg/ml) were stored in a refrigerator (about 4°C), and determined (0—28 days). The drugs in plasma (1 μg/ml) or urine (50 μg/ml) were also determined when incubated at 37°C (0—24 h) or stored at −20°C (0—90 days). The stability of the internal standard in 0.2 *M* phosphate buffer (pH 7.4) was also examined for three months.

Drug administration to human and determination of plasma levels and urinary excretion

AT-2266 was administered orally at a dose of 400 mg to a man. Heparinized blood samples were drawn by venipuncture, and the plasma samples were kept frozen until analysis. The urine samples were collected for 24 h after dosing and also kept frozen until analysis.

RESULTS AND DISCUSSION

Extraction of AT-2266

AT-2266 is an amphoteric compound which is not extractable when non-polar solvents (e.g. *n*-hexane, ether) are used. The pH dependence of the extractability of AT-2266 with chloroform as shown in Fig. 2 shows that only 47% of the compound is extracted at pH 7.0—8.0 when equal volumes of the organic and aqueous phases are used. Therefore, the following extractive derivatization procedure was adopted. AT-2266 was extracted as ethyl carbamate by shaking with chloroform containing 1% of ethyl chloroformate in satisfactory yields (Fig. 2). M-5 was similarly derivatized. This procedure could be utilized for the simple and rapid derivatization of primary and secondary amine [4] or for the separation of tertiary amine from primary and secondary

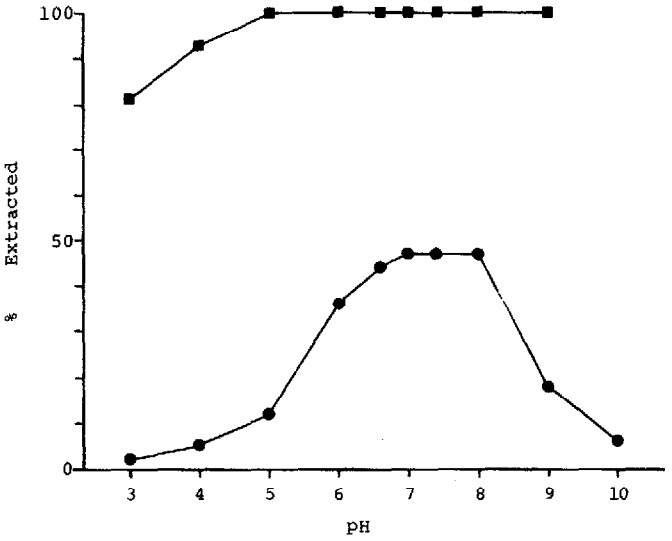

Fig. 2. pH dependence of extraction of AT-2266 (•) and AT-2266 ethyl carbamate (▪). Equal volumes of organic phase and buffered solution (pH 3—10) were used. Organic phase: (•) chloroform, (▪) chloroform containing 1% ethyl chloroformate.

amine [5]. Other metabolites and the internal standard were extracted as the intact form. Moreover, the separation was markedly improved by this procedure. For example, the separation (R_s) of AT-2266 and M-2 was 0.85 and 3.40 before and after derivatization, respectively, when using mobile phase 1.

Determination of AT-2266 and M-2 in plasma

Typical chromatograms of control plasma and plasma containing 1 μg/ml AT-2266 and 0.3 μg/ml M-2 are shown in Fig. 3. With the assay procedure, the peaks of those compounds were found to be separated from those due to control plasma and solvent.

The calibration curve obtained with 0.1—10 μg of AT-2266 in 1 ml of plasma was rectilinear and passed through the origin. The coefficients of

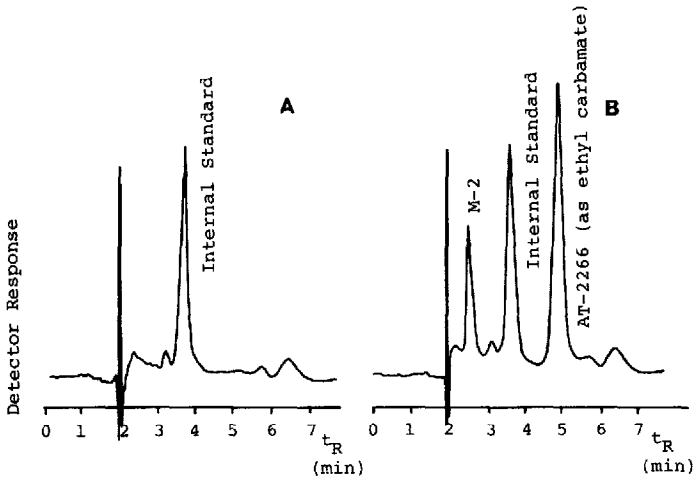

Fig. 3. Typical chromatograms of AT-2266 and its metabolite, M-2, in plasma. (A) Control plasma; (B) AT-2266 and M-2 corresponding to plasma concentrations of 1 and 0.3 μg/ml, respectively. HPLC conditions as described in the text.

variation at 2 and 0.5 μg/ml levels of AT-2266 were 1.9 and 2.8%, respectively. The minimum detectable concentration was 0.01 μg/ml. The calibration curve obtained with 0.03—2.5 μg of M-2 in 1 ml of plasma was also rectilinear. The coefficient of variation was 5.0% at the 0.5 μg/ml level, and the minimum detectable concentration was 0.01 μg/ml. Determinations of AT-2266 and M-2 in plasma obtained on different days were reproducible from day to day.

Determination of AT-2266 and its metabolites in urine

Simultaneous determination of AT-2266 and its five metabolites in urine was possible, but was too time-consuming to be suitable in practice (one chromatogram takes at least 40 min). Therefore, we separated the assay method into two procedures. One was the determination of AT-2266, M-2 and M-5 (procedure U-1), and the other was the determination of M-1, M-3 and M-4 (procedure U-2), which were excreted in minute quantities (less than 1% of the dose).

Fig. 4 shows a typical chromatogram of a urine sample containing AT-2266,

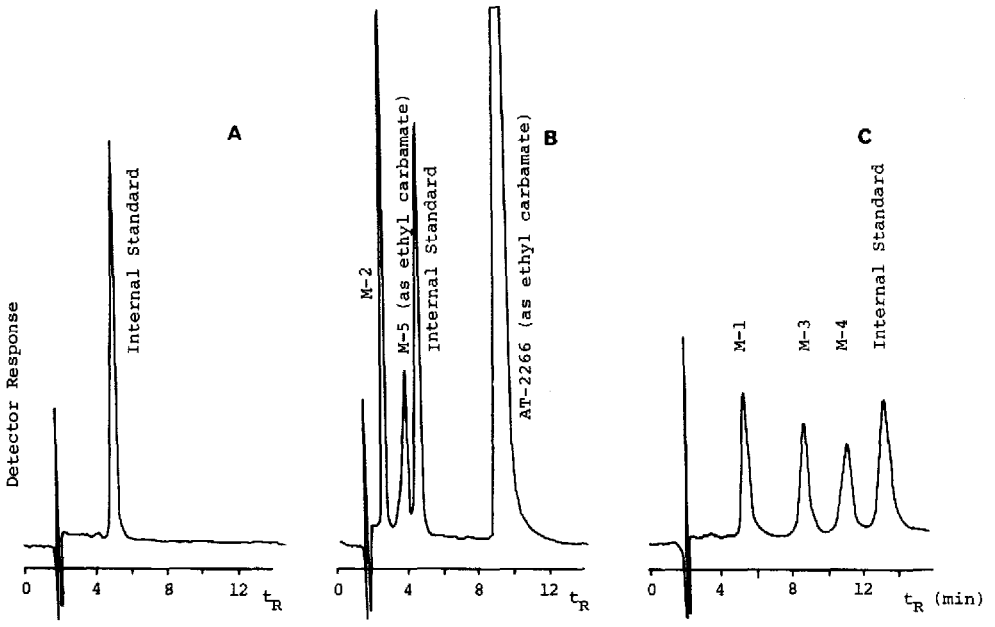

Fig. 4. Typical chromatograms of AT-2266 and its metabolites in urine. (A) Control urine; (B) AT-2266, M-2 and M-5 corresponding to urine concentrations of 51, 13 and 5 μg/ml, respectively; (C) M-1, M-3 and M-4 corresponding to urine concentrations of 5 μg/ml.

M-2 and M-5, and a chromatogram of control urine treated according to procedure U-1.

The calibration curve obtained with 1—500 μg of AT-2266 in 1 ml of urine was a straight line over 500-fold concentration range and passed through the origin. The coefficients of variation at 100 and 20 μg/ml levels of AT-2266 were 1.1 and 1.4% (n = 6), respectively. The minimum detectable concentration was 0.1 μg/ml. The calibration curves of M-2 and M-5 in urine were linear and passed through the origin. The precisions and minimum detectable concentrations were similar to those of AT-2266. Determinations of AT-2266, M-2 and M-5 in urine obtained on different days were reproducible from day to day.

A typical chromatogram of urine containing M-1, M-3 and M-4 treated according to procedure U-2 is shown in Fig. 4. The calibration curves with the same concentration range of 1—20 μg/ml were linear and passed through the origin. The coefficients of variation were 4.2, 2.3 and 3.4% (n = 3) at 5 μg/ml levels of M-1, M-3 and M-4, respectively.

Stability of the compounds

AT-2266 and its metabolites were stable in methanol for at least 28 days when stored in a refrigerator (about 4°C), and the compounds were stable in plasma and urine for at least 24 h at 37°C and 90 days at −20°C. The internal standard was also stable for at least three months in 0.2 M phosphate buffer (pH 7.4) in a refrigerator.

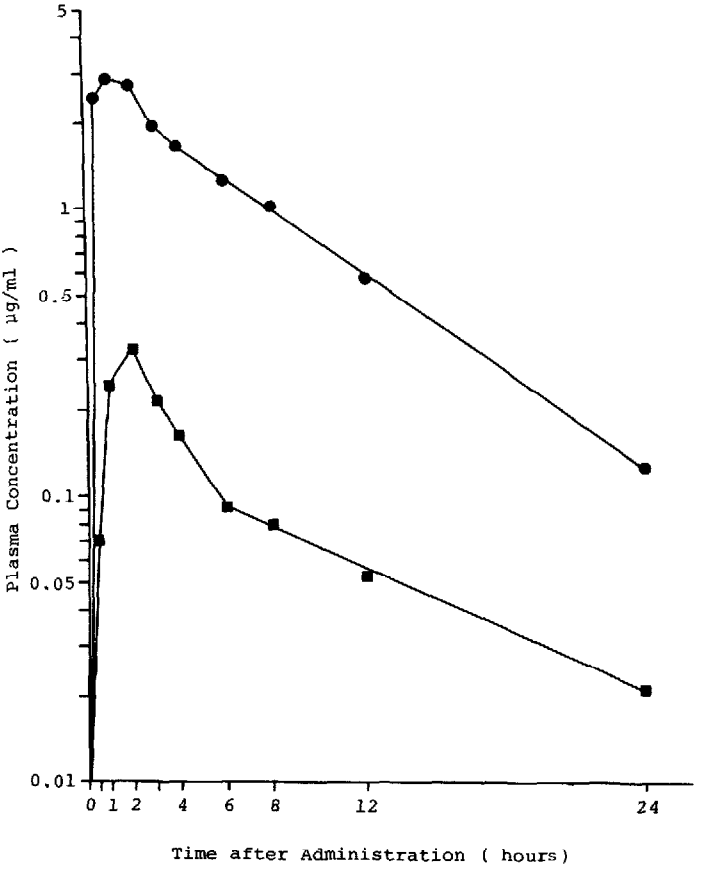

Fig. 5. Plasma levels of AT-2266 (•) and its metabolite, M-2 (▪), in a man following single oral administration at a dose of 400 mg of AT-2266.

TABLE I

24-h URINARY EXCRETION OF AT-2266 AND ITS METABOLITES IN A MAN FOLLOWING SINGLE ORAL ADMINISTRATION OF 400 mg OF AT-2266

Percentage of dose						
AT-2266	M-1	M-2	M-3	M-4	M-5	Total
54.26	0.27	8.11	0.13	0.29	0.99	64.05

Plasma levels and urinary excretion of AT-2266 in man

Plasma levels of AT-2266 and M-2 in a man after single oral dose of 400 mg of AT-2266 are shown in Fig. 5. Plasma levels of the unchanged drug were maximal 1 h after dosing with a level of 2.86 µg/ml, followed by a biphasic decrease with an apparent terminal elimination half-life of 5.3 h. Plasma levels of M-2 were about one-tenth those of the unchanged drug, and the curve was similar to that of the unchanged drug.

Urinary excretion of the unchanged drug and M-2 for 24 h after dosing were

found to be 54.26 and 8.11% of the given dose, respectively, and those of other metabolites were less than 1% of the dose (Table I).

CONCLUSION

We have developed a selective HPLC method for the determination of AT-2266 and its metabolites in plasma and urine. The high specificity and simplicity of the method would permit pharmacokinetic and bioavailability studies in both man and experimental animals.

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REFERENCES

1 J. Matsumoto, T. Miyamoto, A. Minamida, Y. Nishimura, H. Egawa and H. Nishimura, J. Med. Chem., 27 (1984) in press.
2 Y. Sekine, T. Yamaguchi, M. Miyamoto, R. Nakamura, K. Yoshida, A. Minami, S. Nakamura and M. Hashimoto, Chemotherapy. 32 (1984) in press.
3 S. Minami, T. Shono and J. Matsumoto, Chem. Pharm. Bull., 19 (1971) 1426.
4 M. Hashimoto, Y. Sekine, J. Muto, T. Yamaguchi, T. Maeda, Y. Utsui, K. Yoshida, S. Arakawa, N. Shimokawa and K. Shimakawa, Drug Metab. Dispos., 7 (1977) 435.
5 T. Yamaguchi, S. Yoshikawa, Y. Sekine and M. Hashimoto, J. Chromatogr., 173 (1979) 147.