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Note

Determination of ampicillin, amoxicillin, cephalexin, and cephradine in plasma by high-performance liquid chromatography using fluorometric detection

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Several methods for the assay of aminopenicillins [1—8] and aminocephalosporins [9—16] in the body fluids have been studied. These utilize techniques such as fluorometory [1—6, 9—12] and high-performance liquid chromatography with UV detection [7, 8, 13—16]. These methods including our methods, however, are not sufficient for concentrations below about 50 ng/ml of plasma. And in the case of the experiments which study intestinal absorption behavior of these drugs, a more sensitive method of determination in the body fluids is required. Recently, Uno et al. [17], Lebelle et al. [18], and Barbhaiya et al. [19] showed that the structure of the fluorescent degradation products employed in some fluorometric assay procedures, including products obtained by our procedures, had a pyrazine ring as a common structural unit. In this study, we have developed a more sensitive and a reproducible high-performance liquid chromatographic (HPLC) assay for ampicillin, amoxicillin, cephalexin, and cephradine based on the common structure of these fluorescent degradation products obtained by our previous assay procedures.

EXPERIMENTAL

Materials

Ampicillin anhydrous, amoxicillin trihydrate, cephalexin monohydrate, and cephradine dihydrate were kindly supplied by Takeda Chemical Industries, Osaka, Japan, Kyowahakko Kogyo Co., Tokyo, Japan, Shionogi Co., Osaka, Japan, and Sankyo Co., Tokyo, Japan, respectively. Methyl anthranylate was purchased from Wako Pure Chemicals, Osaka, Japan. All the chemicals were of reagent grade and used without further purification.

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Buffer solution

A Sörensen buffer solution (pH 2.5) was prepared as follows. Citric acid (21.0 g) was dissolved in 200 ml of 1 *N* sodium hydroxide solution and the mixture was diluted to 1 liter with distilled water (0.1 *M* disodium hydrogen citrate solution). Then this solution was mixed with 0.1 *N* hydrochloric acid to adjust the pH of the solution to 2.5.

Formation of the fluorescent degradation products in plasma

Ampicillin. Plasma sample (0.5 ml) was added to 4 ml of distilled water in a 10-ml glass-stoppered centrifuge tube. Three milliliters of 10% trichloroacetic acid solution were then added to this diluted plasma sample and the mixture was centrifuged at 800—1000 *g* for 5 min. Three milliliters of the supernatant were pipetted into a test tube containing 0.5 ml of 2 *N* sodium hydroxide solution, then the mixture was allowed to stand for 5 min. Then 0.5 ml of 2 *N* hydrochloric acid was added. To this mixture, 1 ml of 0.1% (w/v) mercury bichloride solution prepared in Sörensen buffer solution (pH 2.5) was added. After 5 min, 2 ml of prewarmed 2/3 *M* disodium hydrogen phosphate solution were added to adjust the pH of the medium to 6.2. The mixture was then warmed at 40° C for 25 min. Then, 6 ml of ethyl acetate saturated with distilled water were added; the mixture was vigorously shaken for 5 min then centrifuged. Five milliliters of the organic layer were pipetted into a brown test tube and evaporated in vacuo. The residue was dissolved in 100 μl of methanol containing internal standard (methyl anthranylate); 20 μl of this solution were injected into the HPLC system.

For the determination in plasma of penicilloic acid, which is a metabolite of ampicillin, 1 ml of distilled water instead of sodium hydroxide and hydrochloric acid, and 0.5 *M* disodium hydrogen phosphate solution instead of 2/3 *M* solution were used; then the same method was followed as described above.

Amoxicillin. Three milliliters of the supernatant obtained from plasma were pipetted into a test tube containing 0.5 ml of 2 *N* sodium hydroxide solution and the mixture was allowed to stand for 5 min. Then 0.5 ml of 2 *N* hydrochloric acid was added. To this mixture, 2 ml of 0.002% (w/v) mercury bichloride solution prepared in 0.5 *M* disodium hydrogen phosphate solution were added to adjust the pH of this mixture to 6.0. A solution of the fluorescent degradation product was obtained by warming this mixture at 50° C for 25 min. After cooling, 6 ml of ethyl acetate saturated with distilled water were added and the mixture was vigorously shaken for 5 min and then centrifuged. Five milliliters of this organic layer were subjected to the method for the determination of ampicillin described above.

For the determination of the penicilloic acid of amoxicillin in plasma, 1 ml of distilled water instead of sodium hydroxide and hydrochloric acid was added to the 3 ml of supernatant. To this mixture, 2 ml of 0.002% (w/v) mercury bichloride solution prepared in 5/12 *M* disodium hydrogen phosphate solution were added to adjust the pH of the mixture to 6.0; then the same method as described above was followed.

Cephalexin. The procedure for the deproteinization of plasma was followed as described above. Three milliliters of the supernatant were pipetted into a

test tube containing 2 ml of 0.1 *M* disodium hydrogen citrate solution. One milliliter of 0.5% (w/v) hydrogen peroxide solution prepared in 0.1 *M* disodium hydrogen citrate solution was then added and the mixture (final pH 2.0) was heated in a boiling water bath for 70 min. This solution was cooled to room temperature and 2 ml of 0.5 *M* disodium hydrogen phosphate solution were added. Seven milliliters of the acetone–chloroform mixture (2:3, v/v) were added and the solution was vigorously shaken for 5 min and then centrifuged. Five milliliters of the organic layer were subjected to the method for the determination of ampicillin as described above.

Cephradine. The procedure for formation of the fluorescent degradation product of cephradine is similar to that of cephalexin except for a few details. Three milliliters of the centrifuged supernatant obtained from plasma were pipetted into a test tube containing 3 ml of 0.1 *M* disodium hydrogen citrate solution. Next, 1 ml of 0.6% (w/v) hydrogen peroxide solution prepared in 0.1 *M* disodium hydrogen citrate solution was added, and the mixture (final pH 2.5) was heated in a boiling water bath for 55 min. This solution was cooled to room temperature and 1 ml of prewarmed 1 *M* disodium hydrogen phosphate solution was added. The medium was then subjected to the method for the determination of cephalexin as described above.

Chromatographic conditions

A liquid chromatograph (Hitachi 638) equipped with a high-pressure sampling valve (638-0801, 1—150 μl) and fluorescence spectrometer (Hitachi 650-60) equipped with a flow cell (635-8001, 18 μl) was used. For the stationary phase, the reversed-phase column (Nucleosil C_{18}, 5 μm, 25 cm × 4 mm I.D., Macherey, Nagel & Co., Düren, G.F.R.) was used, and the column was warmed at 55°C using a constant-temperature water bath circulator. For the determination of ampicillin, cephalexin, and cephradine, a mixture of methanol–distilled water (3:2, v/v) was used as a mobile phase and the effluent was monitored at an excitation wavelength of 345 nm and an emission wavelength of 420 nm. For the determination of amoxicillin, the ratio of constituents of the mobile phase was modified to 55:45 (methanol–distilled water) and the fluorescence measurement was made with the excitation set at 355 nm and emission at 435 nm.

Calibration curve

Standard solutions containing 25, 50, 100, 150, and 250 ng/ml of each drug in distilled water were prepared. One milliliter of standard solution and 3 ml of distilled water were added to 0.5 ml of drug-free plasma and the samples were processed as described above. The ratios of the peak height of each drug to that of methyl anthranylate (internal standard) were used to construct a calibration graph.

RESULTS AND DISCUSSION

HPLC chromatogram

Fig. 1 shows a chromatogram of plasma spiked with 10 ng/ml of cephalexin compared to that of a plasma blank. The fluorescent degradation product of

cephalexin and the internal standard were well separated from endogenous substances. The retention time of the fluorescent degradation products obtained from ampicillin and cephradine were the same as that of the peak obtained from cephalexin. It was also found that the retention time of the fluorescent degradation product of amoxicillin was earlier than those of the other three fluorescent products, and the separation from the endogenous substances was carried out by modifying the ratio of constituents of the mobile phase.

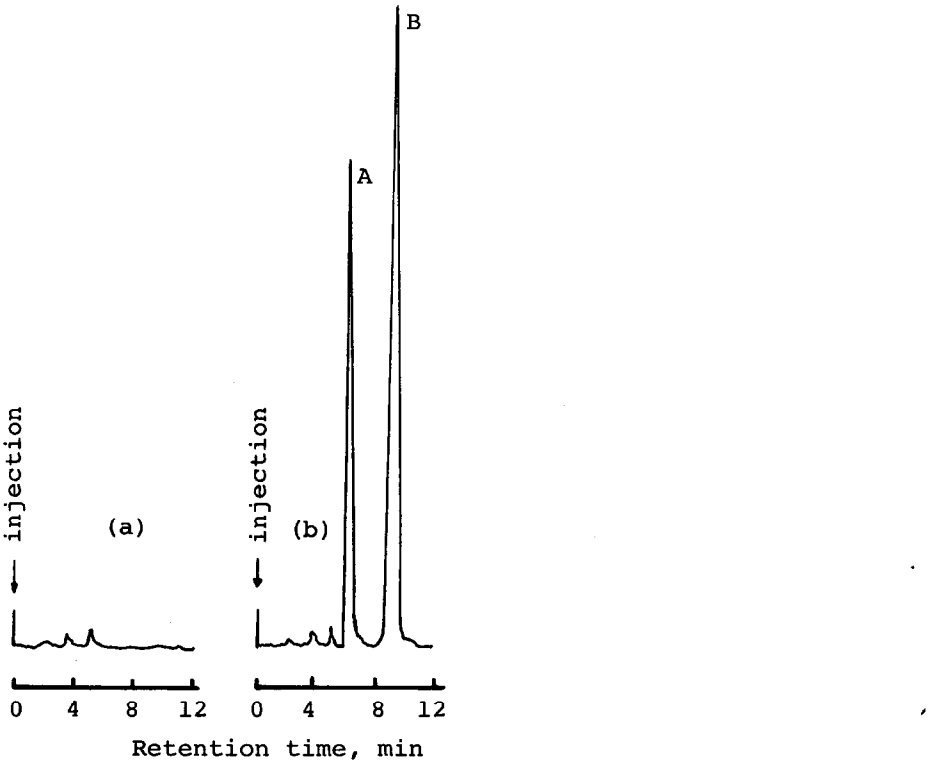

Fig. 1. HPLC chromatograms of (a) blank plasma, and (b) plasma spiked with 10 ng/ml cephalexin.

Selectivity

The calibration curves of peak height ratio for the four fluorescent degradation products were all linear with correlation coefficients of 0.998—0.999. The coefficients of variation of ampicillin and cephalexin at 2 ng/ml plasma were 5.1% (n = 5) and 3.5% (n = 9), respectively. The limit of the determination of the methods was 0.5 ng/ml for ampicillin, 2 ng/ml for cephalexin, and 10 ng/ml for amoxicillin and cephradine.

By using the rat intestinal loop technique for the absorption experiment [20] and this newly developed assay method the concentrations of cephalexin in plasma obtained from the mesenteric vein and carotid artery were determined. The time course of the plasma concentration of cephalexin in the

mesenteric vein after injection of the drug to the loop (150 μM) was 61.2 ng/ml (at 10 min), 80.0 ng/ml (at 15 min), 109.6 ng/ml (at 20 min), 231.4 ng/ml (at 25 min), and 259.5 ng/ml (at 30 min). The concentration of cephalexin in plasma obtained from the carotid artery was 41.9 ng/ml at 30 min.

It is possible to determine the low plasma concentrations of ampicillin, amoxicillin, cephalexin, and cephradine sensitively by the method described in this report. Our results suggest that the method is useful for studying intestinal absorption mechanisms of these drugs and for the determination of these drugs present in body fluids in very small quantities.

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