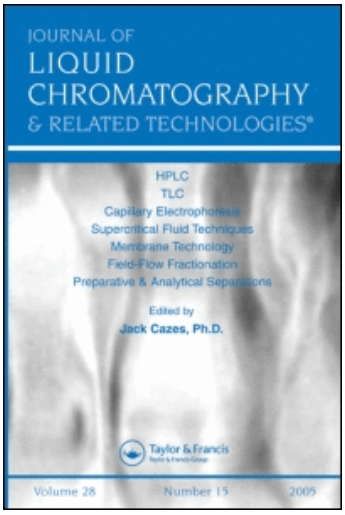

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Simultaneous HPLC Assay of Lenampicillin (Ampicillin Prodrug), Ampicillin, and Marker Compound for High-Throughput in Vitro Assessment of Intestinal Absorption and Metabolism Using Caco-2 Cells

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Abstract: A high performance liquid chromatographic assay method was established to simultaneously determine lenampicillin (ampicillin prodrug), ampicillin, and an absorption marker compound (antipyrine) for the high-throughput *in vitro* assessment of intestinal absorption and metabolism using Caco-2 cells. These compounds were determined using an octyl (C_8) column, mobile phase consisting of 37% methanol and 10 mM NaH_2PO_4 in water (pH 4.5) at a flow rate of 1.5 mL/min, and a UV-detector (200 nm). For lenampicillin, the slope and its S.D. of a typical calibration line going through the coordinate origin (peak area ratio of lenampicillin to internal standard versus lenampicillin concentration from 0.5 μM to 0.5 mM) were 2.91×10^{-3} and 1.83×10^{-5}, respectively. The correlation coefficient (r) of the calibration line in the inter-assays of lenampicillin was 0.9994 (coefficient of variation = 0.04%). For ampicillin, the slope and its S.D. of a typical calibration line going through the coordinate origin (peak area ratio of ampicillin to internal standard versus ampicillin concentration from 0.5 μM to 0.5 mM) were 2.28×10^{-3} and 1.04×10^{-5}, respectively. The correlation coefficient (r) of the calibration line in the inter-assays of ampicillin was 0.9999 (coefficient of

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variation = 0.01%). Lenampicillin absorption and metabolism to ampicillin were shown by this assay method.

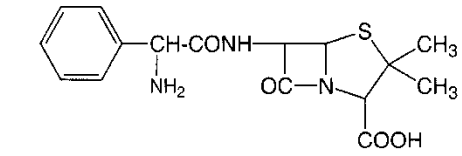
Keywords: Lenampicillin, Ampicillin, Prodrug, Caco-2 cell, High-throughput screening, Absorption

INTRODUCTION

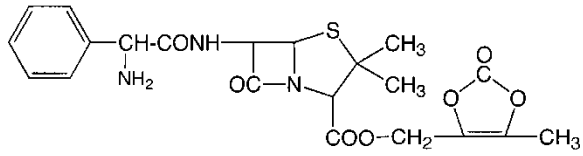
Lenampicillin is a prodrug of ampicillin (Figure 1), and its bioavailability is higher than ampicillin.[1] Although higher lipophilic prodrugs, such as lenampicillin, are designed to improve poor intestinal absorption of active drugs, the details of their mechanism or rational strategy remain unclear. In order to study lenampicillin pharmacokinetics, the simultaneous determination of not only lenampicillin but also its metabolite, ampicillin, should be performed. However, only one study has been reported,[2] and the assay condition was not sufficient for our purpose as described below.

Caco-2 cells are useful to study the intestinal absorption of drug candidates in a high-throughput *in vitro* experiment.[3] However, permeability to the cell monolayer varies, and depends on the conditions for cell culture. For standardization of the drug absorption data, we concomitantly used antipyrine as a well-absorbed marker compound in order to simultaneously assess the absorption (membrane transport) in an *in vitro* experiment using Caco-2 cells. To achieve this study, it is necessary to develop a simultaneous assay of lenampicilin (ampicillin prodrug), ampicillin, and antipyrine.

Therefore, in this study, we established an HPLC assay method using simple isocratic mobile phase to simultaneously determine the compounds contained in the sample in high-throughput *in vitro* absorption experiments of lenampicillin using Caco-2 cells. From the viewpoint of cost, we also



ampicillin



lenampicillin

Figure 1. Structure of ampicillin and its prodrug, lenampicillin.

used methanol as an organic solvent of the mobile phase instead of acetonitrile, which was used in the method by Marzo et al.[2]

EXPERIMENTAL

Chemicals

Ampicillin, antipyrine, and p-nitrophenol were purchased from Wako Pure Chemicals (Tokyo, Japan). Lenampicillin was kindly supplied by Organon Japan (Osaka, Japan). Methanol was purchased from Sigma (St. Louis, USA). All other chemicals were of reagent grade.

HPLC Assay Condition

The HPLC system (Hitachi, Japan) consisted of a pump (655A-11), a UV detector (655A-21) set at 200 nm, an integrator (D-2500), and a TSKgel Octyl-80Ts column (4.6 mm i.d. × 150 mm length, Tosoh, Japan). The mobile phase consisted of 37% methanol and 10 mM NaH_2PO_4 in water (pH 4.5). The flow rate of the mobile phase was 1.5 mL/min.

Absorption Experiment Using Caco-2 Cell Monolayer

Caco-2 cells were cultured according to the previously reported method,[4,5] which followed the method by Hidalgo et al.[3] A solution of lenampicillin and antipyrine dissolved in modified Krebs-Ringer buffer[5] was applied to the apical side of the Caco-2 cell monolayer cultured on Transwell inserts, and modified Krebs-Ringer buffer was applied to the basal side. Then, 100 μL of buffer was periodically sampled from the basal side, and mixed with 20 μL of internal standard (I.S., p-nitrophenol) solution and 26 μL of 0.567% phospholic acid solution in a microtube. Three to eighty μL of the mixture was used in the HPLC assay.

RESULTS AND DISCUSSION

Simultaneous Determination of Ampicillin, Lenampicillin, and Antipyrine

Figure 2 shows a chromatogram of ampicillin, lenampicillin, antipyrine, and p-nitrophenol. Ampicillin, antipyrine, p-nitrophenol, and lenampicillin were eluted in this order, indicating that this assay condition provides simultaneous determination of these compounds.

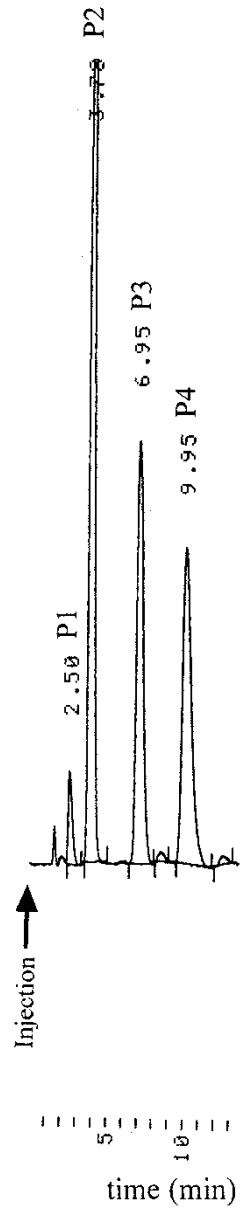


Figure 2. Chromatograms of lenampicillin, ampicillin, antipyrine, and p-nitrophenol (internal standard) determined by HPLC. Peaks: P1, ampicillin; P2, antipyrine; P3, I.S. (p-nitrophenol); P4, lenampicillin. Assay conditions: column, Octyl-80Ts column (4.6 mm i.d. × 150 mm length); mobile phase, 37% methanol and 10 mM NaH_2PO_4 in water (pH 4.5); flow rate of mobile phase, 1.5 mL/min; UV detector, 200 nm.

Standard Curve of Lenampicillin and Ampicillin

For lenampicillin, the slope and its S.D. of a typical calibration line going through the coordinate origin (peak area ratio of lenampicillin to internal standard versus lenampicillin concentration from 0.5 μM to 0.5 mM) were 2.91×10^{-3} and 1.83×10^{-5}, respectively. The correlation coefficient (r) of the calibration line in the inter-assays of lenampicillin was 0.9994 (coefficient of variation = 0.04%) (Table 1). For ampicillin, the slope and its S.D. of a typical calibration line going through the coordinate origin (peak area ratio of ampicillin to internal standard versus ampicillin concentration from 0.5 μM to 0.5 mM) were 2.28×10^{-3} and 1.04×10^{-5}, respectively. The correlation coefficient (r) of the calibration line in the inter-assays of ampicillin was 0.9999 (coefficient of variation = 0.01%) (Table 1). These results indicate that this HPLC assay method is appropriate to determine ampicillin and lenampicillin.

HPLC Assay of Samples from an in Vitro Caco-2 Cell Experiment

Figure 3a–c show chromatograms of the standard solution (a) and samples (b and c) from an *in vitro* absorption experiment using Caco-2 cells. Ampicillin, antipyrine, p-nitrophenol, and lenampicillin were eluted in this order from the column (Figure 3a). Ampicillin, antipyrine, and lenampicillin were not detected in samples from an *in vitro* experiment at time 0. In contrast, these compounds were detected in samples of the basal side 20 min after incubation, indicating that lenampicillin and antipyrine were absorbed across the Caco-2 cell monolayer. In addition, a portion of lenampicillin was metabolized to ampicillin during absorption across Caco-2 cells. These results indicate that the HPLC assay condition in this study is applicable for the assessment of *in vitro* absorption and metabolism of lenampicillin.

Although Marzo et al.[2] used acetonitrile for the HPLC assay of lenampicillin, in this study, methanol was used, because of the lower cost of methanol than acetonitrile. The combination of a methanol and octyl column makes it possible to keep the assay time within 10 min. The correlation coefficient of the calibration line and detection limit (0.5 μM at highest) of lenampicillin and ampicillin were sufficient to assess lenampicillin absorption

Table 1. Correlation coefficient for HPLC assay of ampicillin and lenampicillin

Drug	Mean	S.D.	C.V. (%)	n
Ampicillin	0.9999	0.0001	0.01	3
Lenampicillin	0.9994	0.0004	0.04	4

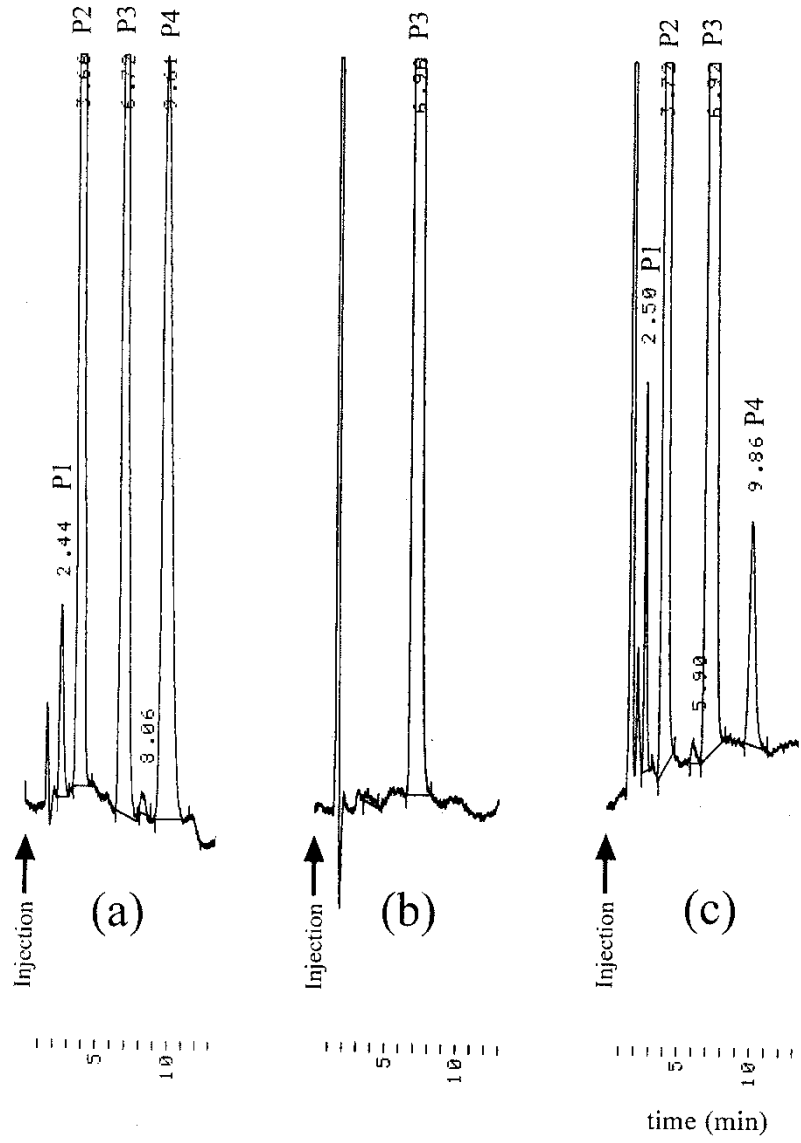


Figure 3. Chromatograms of samples from the absorption experiment using Caco-2 cells. (a) standard solution, (b) sample (basal solution) from an in vitro Caco-2 cell absorption experiment at time 0, and (c) sample (basal solution) from an in vitro Caco-2 cell absorption experiment after 20 min incubation. Peaks: P1, ampicillin; P2, antipyrine; P3, I.S. (p-nitrophenol); P4, lenampicillin. Assay conditions: column, Octyl-80Ts column (4.6 mm i.d. × 150 mm length); mobile phase, 37% methanol and 10 mM NaH_2PO_4 in water (pH 4.5); flow rate of mobile phase, 1.5 mL/min; UV detector, 200 nm.

(membrane transport) and metabolism in an *in vitro* experiment using Caco-2 cells. The quantitative and kinetic study of lenampicillin absorption and metabolism is now in progress.

In conclusion, the HPLC method using simple isocratic mobile phase is useful to simultaneously determine lenampicillin, ampicillin, and antipyrine in samples from a high-throughput *in vitro* absorption experiment of lenampicillin using Caco-2 cells.

ACKNOWLEDGMENT

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