Examination of Stability of Anticonvulsants in a Protease Solution and Assay of Anticonvulsants in Hairs

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For analyzing the concentrations of drugs in hairs, a new method of digestion of hairs with Biopurse®, a protease obtained from Bacillus subtilis, was examined. The concentrations of drugs in hairs were then determined in order to examine the usefulness of the protease for the digestion of hairs.

The stability of five anticonvulsants in the protease solution was maintained over a 12-h period. In the clinical tests, the concentrations of the drugs in hairs obtained from patients who were taking anticonvulsants for a long time were determined. The concentration of phenobarbital in hairs in 10 patients taking phenobarbital ranged from 194 to 5020 ng/10 mg with a mean of 578 ng/10 mg, and the concentration of phenytoin in hairs in 6 patients taking phenytoin ranged from 44 to 299 ng/10 mg with a mean of 115 ng/10 mg. From these results, the transfer of phenobarbital and phenytoin from circulating blood into hairs was confirmed, and the usefulness of Biopurse® for the digestion of hairs was proved.

Key words: hair analysis; anticonvulsant; protease

For rational drug treatment in clinical medicine, therapeutic drug monitoring (TDM) is needed. In TDM, serum or urine samples are mainly used. Recently, the usefulness of assay of drugs in scalp hair (hair analysis) in TDM has been reported by Uematsu et al.1–3 The advantages of hair analysis include much less pain when hair samples are collected than that experienced when blood is collected, and the additional provision of information concerning medication history, i.e., the pattern of individual drug use from months to years.4–6 Although hair analyses for drugs of abuse have been widely practiced,7,8 a problem in these studies is the method of extraction. To use the method of extraction. To use the method of extraction. Up to now, three methods of extraction have been reported.9,10 1) Extraction by an organic solvent after hairs are digested by a strong alkaline solution, 2) extraction by a neutral organic solvent, and 3) extraction by acid methanol. There are some problems in these methods of extraction. Primarily, on extraction by an alkaline or acid solution, the stability of the drugs poses an important problem. In this study, the usefulness of a protease obtained from bacteria (Biopurse®) was examined.

MATERIALS AND METHODS

Materials: Biopurse® was generously supplied from Nagase Biochemical Industry Co., Fukuchiyama, Kyoto. This protease was separated and purified from an incubation solution of Bacillus subtilis. The optimum pH condition for enzymatic action is known to be around 8–10.

A formula examined for the digestion of hairs was used in this study.10 The protease solution was 10000 U Biopurse/ml, 5% with respect to both thiglycolate and glycine and 1% with respect to a nonionic surfactant (Emargon A-60) in 0.1 M borate buffer, pH 8 or 10. Phenytoin, ethotoin and zonisamide were generously supplied from Dai nippon Pharmaceutical Co., Osaka, and hexobarbital, phenobarbital and carbamazepine were purchased from Tokyo Chemical Industry Co., Tokyo. MethElute®, a methylating agent for on-column derivatization of drugs in gas chromatography, which was labeled to contain a 0.2 M solution of trimethylaminium hydroxide in anhydrous methanol, was purchased from Pierce Chemical Co., Illinois, U.S.A. Organic solvents were purchased from Wako Pure Chemical Ind., Osaka, and were distilled prior to use. Other chemicals were of reagent grade and purchased from Wako Pure Chemical Ind.

Stability of Drugs in Buffer Solutions Because the optimum pH for the enzyme has been shown to be 8–10, the stability of drugs in solutions at pH 8 or 10 was examined. The concentration of respective drugs was 1 μg/ml. Respective drugs were added into a solution at the respective pH. These solutions were incubated at 37°C, and samples were withdrawn from these solutions periodically. The change in UV absorbance of the samples was determined spectrophotometrically.

Stability of Drugs in the Protease Solution Five anticonvulsants (phenobarbital, phenytoin, ethotoin, zonisamide, and carbamazepine) were added to the protease solution, the solutions were then incubated at 37°C, and samples were obtained from these solutions according to a protocol. The concentrations of respective drugs were 60 μg/ml (phenobarbital), 7.5 μg/ml (phenytoin), 2.5 μg/ml (carbamazepine), 50 μg/ml (ethotoin) and 5 μg/ml (zonisamide). The differences in concentrations of the drugs did not have any effect on the stability of the drugs. To a 200-μl portion of the sample in a 10-ml glass test tube, 1 ml of chloroform containing hexobarbital as an internal standard material was added. The sample was agitated for 10 min by a horizontal shaking apparatus, and after centrifugation, the upper aqueous layer was aspirated off. The chloroform layer which remained was then transferred into a 10-ml glass sample tube and evaporated to dryness in vacuo at 40°C. The residue was redissolved in methanol and injected into the HPLC system. HPLC conditions were as follows:

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The ODS column: Lichrospher® RP-18, 250 mm × 4.6 mm i.d. particle size 5 μm, Cica-Merck, Tokyo. Elution solvents: 0.1 M acetate buffer, pH 4.0: acetonitrile = 70:30 for phenobarbital analysis, distilled water: acetonitrile = 65:35 for phenytoin, carbamazepine and ethotoin analysis, and 1% acetic acid: isopropanol: acetonitrile = 70:11:10 for zonisamide analysis. Flow rate: 1 ml/min for phenobarbital, carbamazepine and phenytoin, 0.8 ml/min for ethotoin, and 0.7 ml/min for zonisamide. Column temperature: 50°C. Monitoring by UV detector: 240 nm for phenobarbital, 230 nm for phenytoin, carbamazepine and ethotoin and 285 nm for zonisamide.

Clinical Study Psychiatically handicapped patients taking anticonvulsants for a long time participated in this study after informed consents were obtained from their guardians, and their hair samples were collected for the determination of phenobarbital and phenytoin. Blood samples were obtained from each patient on the same day as the sampling of hairs.

Digestion and Extraction Procedures for the Hairs The hair samples of patients were washed with distilled water three times. 10 mg of washed and dried hair was weighed. The transfer of drugs to water during a washing process was considered minimal in this study. The hairs were then cut down to 1 cm, and these cut hairs were added to 1 ml of Biopurase® solution and incubated at 37°C for 12 h. Then, to the digested hair solution in a 10-ml glass test tube, were added 1 ml of the saturated solution of sodium chloride and 1 ml of chloroform containing hexobarbital as an internal standard material. These mixtures were agitated for 10 min with a horizontal shaking apparatus. After centrifugation, the upper aqueous layer was aspirated off. The chloroform layer which remained was then transferred into a 10-ml glass sample tube and evaporated to dryness in vacuo at 40°C. The residue was redissolved in 25 μl of MethElute®, and a 1-μl aliquot of the resultant solution was injected into the GC-MS system.

GC-MS Condition A JEOL DX303-DA5000 gas chromatograph-mass spectrometer (GC-MS) system was employed under the following conditions. A GC part of the GC-MS system was equipped with a 1.2 mm × 3 mm (i.d.) glass column of 3% OV-17 on GasChrom Q (Gasukuro Kogyo Inc., Tokyo). The flow rate of helium: 50 ml/min. The temperatures of the injection port, transfer line to the mass spectrometer, and the ion source of the mass spectrometer was 250°C. The column temperature: 230°C. Mass spectra were obtained in an electron impact mode at an electron energy of 70 eV. Selected ion monitoring: m/z 232, 235 and 280 for phenobarbital, hexobarbital and phenytoin, respectively. Peak area ratios of the drug to the internal standard material were calculated to obtain the standard curves.

RESULTS AND DISCUSSION

When the hairs were incubated in the protease solution at 37°C for 30 min, the hairs were broken down below 5 mm, were dispersed like powder after 5 h, and then dissolved completely after 12 h.

1) Stability of Anticonvulsants in Buffer Solutions at pH 8 or 10 The UV absorbance of zonisamide, carbamazepine and phenytoin did not change in buffer solutions at pH 8 or 10. The absorbance of phenobarbital and ethotoin changed after 24 h in solution at pH 10, whereas their absorbance did not change in solution at pH 8 for 96 h. Therefore, an aqueous solution at pH 8 was selected for the examination of drug stability in the protease solution.

2) Stability of Anticonvulsants in Protease Solution The stability of drugs in protease solutions is shown in Figs. 1 and 2. The concentrations of the respective drugs at time 0 are set as 100%, and the percentage change of the drug remaining is shown. In phenobarbital, 100% of phenobarbital remained for 24 h, but 90% of phenobarbital remained after 32 h. In phenytoin, carbamazepine and zonisamide, 100% of drugs remained after 32 h. In ethotoin, 100% of the drug remained for 12 h, but 95% and 60% remained after 24 and 40 h, respectively. These results reflect the poor stability of phenobarbital and ethotoin in aqueous alkaline solution. Because stability in the protease solution for the first 12 h was confirmed for all drugs examined, Biopurase® was proved useful for the digestion of hairs prior to the determination of drug concentrations in hairs.

3) Determination of Concentrations of Phenobarbital and Phenytoin in Hairs Concentrations of phenobarbital and phenytoin in hairs obtained from patients are shown in Tables 1 and 2. GC-MS was employed in this study because it provides more sensitivity and clearer separation among peaks than HPLC. Recovery values were 83% in 10 μg/ml of phenobarbital and 84% in 25 μg/ml of phenytoin. The coefficients of variation for quantitation were below 7%.

Fig. 1. Stability of Carbamazepine, Phenobarbital and Phenytoin during Hydrolysis in the Protease Solution at 37°C
Mean ± S.E.M., n = 4.
Fig. 2. Stability of Ethotoxin and Zonisamide during Hydrolysis in the Protease Solution at 37°C
Mean ± S.E.M., n = 4.

Table 1. Concentrations of Phenobarbital in Serum and Hair Samples

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Serum (µg/ml)</th>
<th>Hair (ng/10 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.6</td>
<td>194</td>
</tr>
<tr>
<td>2</td>
<td>23.1</td>
<td>872</td>
</tr>
<tr>
<td>3</td>
<td>8.3</td>
<td>823</td>
</tr>
<tr>
<td>4</td>
<td>16.8</td>
<td>1097</td>
</tr>
<tr>
<td>5</td>
<td>17.4</td>
<td>411</td>
</tr>
<tr>
<td>6</td>
<td>22.5</td>
<td>653</td>
</tr>
<tr>
<td>7</td>
<td>18.1</td>
<td>652</td>
</tr>
<tr>
<td>8</td>
<td>23.5</td>
<td>780</td>
</tr>
<tr>
<td>9</td>
<td>36.2</td>
<td>5020</td>
</tr>
<tr>
<td>10</td>
<td>10.3</td>
<td>293</td>
</tr>
</tbody>
</table>

Table 2. Concentrations of Phenytoin in Serum and Hair Samples

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Serum (µg/ml)</th>
<th>Hair (ng/10 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>78.2</td>
</tr>
<tr>
<td>2</td>
<td>12.5</td>
<td>103.5</td>
</tr>
<tr>
<td>3</td>
<td>6.8</td>
<td>79.2</td>
</tr>
<tr>
<td>4</td>
<td>9.4</td>
<td>83.3</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>298.9</td>
</tr>
<tr>
<td>6</td>
<td>0.8</td>
<td>43.9</td>
</tr>
</tbody>
</table>

Linear relationships were obtained in the ranges from 50 ng/ml to 5 µg/ml in phenobarbital and from 50 to 500 ng/ml in phenytoin. As shown in Table 1, the concentration of phenobarbital in hairs ranged from 194 to 5020 ng/10 mg with a mean of 578 ng/10 mg; on the other hand, the serum concentration ranged from 7.6 to 36.2 µg/ml in ten patients.

As shown in Table 2, the concentration of phenytoin in hairs ranged from 44 to 299 ng/10 mg with a mean value of 115 ng/10 mg in six patients, whereas the serum concentration ranged from 0.8 to 15 µg/ml. From these assays, the transfer of phenobarbital and phenytoin from the blood to hairs was proved. The correlation of concentrations of drugs in hair with those in blood was analyzed by linear regression, but little correlation was obtained. Although blood and hair were concomitantly collected, because the end parts of hairs were collected, there is much difference between time when the end part of the hair grew and the time when the blood sample was drawn.

Usefulness of the protease (Biopurase®) treatment for the hydrolysis of hairs over other treatment procedures may be cited. Namely, 1) in alkaline digestion, some error has to be considered in some drugs which are hydrolyzed at an alkaline pH, 2) in solvent extraction, the extent of extraction with organic solvents has to be examined, 3) in acid methanol extraction, some error has to be considered in some drugs which are hydrolyzed at an acidic pH. The present method offers the extraction of drugs from hair matrices without much risk of hydrolysis of drugs which are susceptible to alkaline or acid treatment. Thus, concentrations of phenobarbital and phenytoin in hair samples were determined and the transfer of drugs to hairs was confirmed.

The concentrations of drugs in hairs and those in serum, however, showed little correlation, although the latter shows concentrations at a sampling time but not a medication history. There are only limited numbers of reports on the correlation. The concentration of haloperidol in hairs and doses showed correlation \(^1\) whereas the concentrations of 5-fluorouracil in hairs and doses showed little correlation \(^5\). In this study, hairs were collected from various parts of the head. Depending on the parts of head on which hairs are grown, the difference in growth rate and melanin content, which are expected to be an important factors in transfer of drugs to hairs, \(^11\) has to be considered. To improve the correlation, the area of the head where hairs are cut and the sampling time of the blood after administration should be enforced in the protocol. If we design the protocol carefully, we expect the correlation between the concentrations of drugs in hairs and those in serum to be improved.

There are few reports which showed a transfer of phenobarbital or phenytoin to hair after confirmation of the stability of drugs during extraction. The present study demonstrates the presence of phenobarbital and phenytoin in hair in patients whose blood concentrations are in a therapeutic range.

In addition, we are considering the application of hair analysis to the diagnosis of drug dependence, the assessment of clinical pharmacokinetics in a fetus or infant, and the assessment of compliance. At present, the assessment of compliance depends only on questioning patients. This method, however, is indirect and the results are doubtful. Since the use of concentration in hairs is a direct assessment of compliance, its importance should be appreciated. When drugs are taken by pregnant women, the fetus is exposed to drugs, but assessment of
the influence of drugs on the fetus is difficult. There is a possibility that the determination of concentrations of drugs in hairs in newborns can become a useful way of assessment of the exposure of the fetus to drugs.

REFERENCES AND NOTES

10) Nagase Biochemical Laboratories, Fukuchiyama, Kyoto, Japan.