Hair Analysis for Pharmaceutical Drugs.

I. Effective Extraction and Determination of Phenobarbital, Phenytoin and Their Major Metabolites in Rat and Human Hair

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In order to establish an analytical method for the determination of phenobarbital (PB), phenytoin (PPH) and their hydroxylated metabolites in hair, animal model experiments were performed. Five male dark-agouti pigmented rats, aged 5 weeks, were intraperitoneally and orally administered PB or PPH independently at 25 mg/kg once a day for 5 successive days. The growing back hair was collected 15 d after the first administration. Four typical extraction methods, using NH4OH–methanol–acetonitrile, TFA–methanol–acetonitrile, 1 M sodium hydroxide and proteinase K, were evaluated using the rat hair samples containing PB or PPH. Methanol–acetonitrile–NH4OH (10 : 10 : 1) was the best extraction method from all aspects, such as high extraction efficiency and low noise. The analytes in the extract were methylated in acetonitrile with 20% tetramethylammonium hydroxide and methyl iodide at 70 °C for 10 min. After purification with Bond Elut Certify, the methylated products were analyzed by GC-MS. From rat hair, PB, p-hydroxy PB, PPH and p-hydroxy PPH were detected at average concentrations of 26.9, trace, 4.2 and 0.4 ng/mg with an intraperitoneal (i.p.) injection, and at 30.9, trace, 4.0 and 0.4 ng/mg with oral administration, respectively. There was little difference in hair concentrations between i.p. injection and oral administration. This method was applied to the head hair of two patients who orally took toxic amounts of PB and two volunteers who orally took 100 mg of PPH daily for 5 d. The hair concentrations of PB in the two patients were 16.2 and 14.7 ng/mg, and those of PPH in the two volunteers were 3.3 and 0.1 ng/mg.

Key words hair; phenobarbital; phenytoin; drug poisoning; drug incorporation into hair

In recent years, various poisoning cases involving pharmaceutical drugs have occurred in Japan. To determine the drugs involved, biological samples are analyzed, usually urine or blood. However, there are some cases in which such typical samples are hard to obtain or the drugs have already disappeared from the urine and/or blood due to the lapse of time after taking the drugs. In those cases, hair generally becomes the next choice as an alternative sample. Nevertheless, very few studies of hair analysis to detect pharmaceutical drugs have been reported. Therefore, it is currently difficult to detect and determine pharmaceutical drugs in hair due to the lack of literature concerning hair analysis for them. The authors of this group have reported hair analysis studies, especially for abused drugs.3) As a part of our study, we are also investigating hair analysis for pharmaceutical drugs in order to establish analytical methods for them, and are applying these to human hair samples and hair pharmacokinetic studies with animal models.

At present, phenobarbital (PB) and phenytoin (PPH) are the most popular pharmaceutical drugs used as anti-epileptics. Moreover, they are both drugs which may cause poisoning by their overdose.3) So far there have been few reports describing hair analysis for PB and PPH. Since 1981, the detection of PB in hair by GC-MS4–7) has been reported for the purposes of forensic interests and therapeutic monitoring. Mei and Williams8) reported the determination of phenytoin in human hair by HPLC for the compliance of patients during anti-epileptic therapy.

In these reports, no investigation of extraction efficiency and no comparison between different extraction methods was presented. In this study, our aims were to compare four typical extraction methods, to clarify the extraction efficiency, accuracy and reproducibility, and to investigate the hair incorporation rates for PB and PPH using animal model experiments. In addition, our method was applied to hair samples obtained from humans who took PB or PPH.

MATERIALS AND METHODS

Materials

Chemicals and Materials: Phenytoin (PPH), 5-(4-methylphenyl)-5-phenylhydantoin (internal standard; MPPH), phenobarbital (PB), p-hydroxyphenobarbital (HPB) and p-hydroxyphényl phenylhydantoin (HPPH) were obtained from Sigma (St. Louis, MO, U.S.A.), and 5-(4-hydroxyphenyl) hydantoin (internal standard; HPH) from Tokyo Chemical Industry (Tokyo, Japan). Pentadecuterated PB (internal standard; PB-d5) was purchased from Radian (Austin, TX, U.S.A.). Other reagents and organic solvents were purchased from Wako Pure Chemical Industries (Osaka, Japan). A TC-1 capillary column was obtained from GL Sciences (Tokyo, Japan), and Bond Elut Certify columns from Varian (Harbor City, CA, U.S.A.).

Instrumentation: For the analysis of PPH, PB and their metabolites, GC-MS in the electron impact (EI) mode was used. GC-MS analyses were carried out using a Hewlett Packard 5890 series-II gas chromatograph equipped with a 7673A autosampler and MSD 5971. The gas chromatography (GC) was carried out with a 30 m×0.25 mm I.D., 0.25 μm cross-linked methylsilicone fused silica TC-1. The injection port temperature was 200 °C (splitless mode) and helium was the carrier gas (4.5 psi head pressure). The oven temperature was held at 90 °C for 0.5 min following injection, and was programmed to reach 280 °C at a rate of 20°C/min. Drugs in biological specimens were investigated by monitoring the selected ions as follows: dimethyl-PPH m/z: 194, 203, 280; dimethyl-PB m/z: 175, 232, 260; trimethyl-HPPH m/z: 224, 233, 310; trimethyl-HPH m/z: 233, 261, 290.

For quantitative analysis, drug concentrations in the bio-
logical specimens were calculated using the peak-area ratios of the ions monitored for the analytes and their internal standards (ISs); for dimethyl-PPH (m/z 280) with dimethyl-MPPH (m/z 294), trimethyl-HPPH (m/z 310) and trimethyl-HPB (m/z 261) with trimethyl-HPH (m/z 233), and for dimethyl-PB (m/z 232) with dimethyl-PB-d5 (m/z 237).

The limit of detection for PPH and PB was 0.03 ng/mg, and that for HPPH and HPB was 0.1 ng/mg.

**Animal Experiments** Before drug administration, the back hair of rats was shaved with an animal electric shaver. Five male dark-agouti (DA) pigmented rats, aged 5 weeks, were intraperitoneally and orally administered PPH or PB independently at 25 mg/kg daily for 5 successive days. The regrown hair on the back was collected 15 d after the first administration. This hair sample was used to investigate the reproducibility and accuracy of our method.

**Human Hair Sample** Phenobarbital: Hair samples containing hair roots were obtained by plucking them from the heads of two patients (male, age 48; female, age 51) who took overdoses of Vegetamin A (a sedative of Shionogi Co., Ltd. containing 40 mg of PB in a tablet) and were taken to the emergency room. The 1 cm root side of the hair was used for analysis.

Phenytoin: Two male volunteers (ages 38 and 55) took 100 mg of phenytoin granule daily for 5 d. Hair was cut near the skin from the vertex region by scissors 7 d after the last dose. The 5 mm root side of the hair was used for analysis.

These human subjects gave informed consent prior to drug administration.

**Investigation for Optimum Extraction from Hair Sample** Standard Solutions: Stock solutions for standard curves were prepared for each drug at 1 mg/ml in methanol and stored at 4 °C. From these solutions, two sets of working solutions were prepared, one for plasma and one for hair and stored at 4 °C. From these solutions, two sets of working curves were prepared for each drug at 1 mg/ml in methanol.

**Accuracy and Reproducibility of the Analytical Method** To test the accuracy of the method, 10 mg of control rat powdered hair spiked with 100, 100, 10 and 10 ng of PPH, PB, HPPH and HPB, respectively, was used. The accuracy of the basic methanolic extraction method was calculated by triplicate analyses using calibration curves of the standard solutions.

For reproducibility testing, hair samples obtained from the animal experiments were used. The reproducibility of the basic methanolic extraction method was determined by analyzing four hair samples (10 mg each).

**Effect of Administration Route on Hair Concentration** The hair concentrations of PB and PPH resulting from intraperitoneal (i.p.) administrations (25 mg/kg intraperitoneally for 10 successive days. Blood samples were collected 5, 15, 30, 60, 120 and 360 min after the administration, and plasma samples were prepared in the usual manner. The areas under the concentration vs. time curves (AUCs) were calculated by the trapezoidal rule over the time of measurement, with the remainder of the curve estimated as $\beta \cdot C_{\text{last}}$ where $C_{\text{last}}$ is the concentration of the last observed time point and $\beta$ is the terminal rate constant. After shaving the back hair just before the first administration, the newly growing hair samples were collected 4 weeks after the first administration. The ratio of drug concentration in the
newly grown hair to AUC is regarded as the ICR.

RESULTS AND DISCUSSION

As shown in Fig. 1, it is known that PB is metabolized to HPB and PPH to HPPH as major metabolic pathways. Therefore, we chose these hydroxy metabolites as secondary makers indicating drug intake in hair from blood.

Comparison of Extraction Efficiency between 4 Typical Methods

In an attempt to improve the extraction efficiency of PB, PPH and HPPH from hair, we compared the efficiency of 4 typical extraction methods, including conventional and new methods for PB, PPH and HPPH, using hair samples obtained from rats administered with PB and PPH independently. A total of 4 methods (Proteinase K, methanol–acetone–ammonia, methanol–acetone–TFA, 1 M NaOH) were compared for extraction efficiency. Figure 2 shows the extraction efficiency of PB, PPH and HPPH from rat hair by the 4 methods, with significant difference. The method which yielded the highest recoveries of drugs and had no interfering peaks was methanol–acetone–ammonia extraction overnight. Proteinase K is used for the mild degradation of the hair fiber structure. This method resulted in relatively high recoveries of PB and PPH from hair, but it brought about an interfering peak around the peak of PB and it led to less cost performance than the other methods. The general alkaline digestion method with NaOH is applicable to alkaline stable compounds in hair, but the recovery of the hydroxylated metabolite (HPPH) was significantly low, probably because of its instability in a strongly alkaline media. The extraction efficiency for PPH from hair samples using methanol–acetone–TFA was fairly good, but it was clearly lower than that of methanol–acetone–ammonia for HPPH and PB. Based on these overall results, methanol–acetone–ammonia was used for subsequent experiments as the extraction solvent.

Derivatization and Clean up Procedures

Further clean up procedures were needed to obtain clean chromatograms and to protect against the rapid decay of columns due to the co-extracted hair ingredients. After the reconstituted residue from the methanol–acetone–ammonia extraction mentioned above was derivatized with 20% TMAH and methyliodide in acetonitrile, the reaction mixture was cleaned with Bond Elut Certify. As a result of the recovery experiments using the hair spiked with the target drugs, solid phase extraction was the best choice in terms of clean up, compared to liquid–liquid extraction.

Accuracy and Reproducibility of This Method

The accuracy of this method was evaluated using control rat hair samples spiked with PB and PPH at 10 ng/mg, and HPB and HPPH at 1 ng/mg. As shown in Table 1, the hair concentrations of PB and PPH were determined within a 10% range of error, while those of the hydroxy metabolites were determined in the range of 11—18% error. These large errors were probably caused by the poor reactivity of methylation to the hydroxy metabolites. It is considered that they would be reduced with the deuterated target drugs.

The reproducibility of this method was evaluated using hair samples of rats orally administered PB or PPH at 25 mg/kg 5 times for 5 d. The hair concentrations of PB were determined at a very low standard deviation (2.3%), possibly
because of the use of deuterated PB as the IS, followed by those of PPH (5.5%). In contrast, the standard deviations of hydroxy metabolites were relatively high (7.7 and 10.0%).

Detection of PB, PPH and \(p\)-Hydroxylated Metabolites in Rat Hair

This study was initiated with the detection of parent compounds and hydroxylated metabolites in the hair of rats administered PB and PPH. It was confirmed that there were no peaks around the retention time (8.75 min) of methylated PB on the GC-MS chromatogram from the methylated extracts of control rat hair (Fig. 3A). From the hair of rats administered PB, a peak at 8.75 min was detected (Fig. 3B), which was identical with that of standard methylated PB on the chromatogram and had the principal ions (\(m/z\) 260, 232 and 175) as methylated PB. In the same manner, methylated HPB was detected by a comparison between the control and dosed rat hair at 9.99 min (see Fig. 4).

It was confirmed that there were no peaks around the retention time (10.46 min) of methylated PPH on the GC-MS chromatogram of the methylated extracts from the control rat hair (Fig. 5A). The GC-MS chromatogram of the methylated extracts from the hair of rats administered with PPH is shown in Fig. 5B. A peak was detected at 10.46 min, which was identical with that of standard methylated PPH on the chromatogram and had the same principal ions (\(m/z\) 280, 203 and 194). In the same manner, methylated HPPH was confirmed by comparison between the control and dosed rat hair at 11.84 min (see Fig. 6).

Difference in Hair Concentrations between Intraperitoneal and Oral Doses

The rat hair concentrations of PB and PPH resulting from i.p. injections (25 mg/kg×5) were compared with those from oral administration (25 mg/kg×5). As shown in Table 3, the average hair concentration of PB from i.p. injections was 31.8 ng/mg, while that from oral administrations was 30.9 ng/mg. The average hair concentration of PPH from i.p. injection was 4.2 ng/mg, while that by oral
administration was 4.0 ng/mg. These data show that there was little difference in hair concentration between i.p. injection and oral administration in the case of PB and PPH.

**Drug Incorporation Rate into Hair (ICR)**

The rats were intraperitoneally administered PB and PPH daily at 25 mg/kg for 10 d \((n=3)\). On day 28 after the first administration, the newly grown back hair was collected and then analyzed.

Our group has proposed\(^{14,17}\) an ICR based on the ratios of hair concentrations and plasma \(AUC\) as an index of blood-hair pharmacokinetics. These theories are, however, only general guidelines for prediction, as other factors influence incorporation and binding in hair.

According to our previous studies,\(^{14}\) the ICRs of methamphetamine and cocaine were 0.29 and 3.58, while benzyloleconbine and tetrahydrocannabinolic acid show low ICRs (0.003 and 0.0005). The ICRs of PB and PPH were determined to be 0.0014 and 0.0016, respectively. Those ICRs are much smaller than those of other basic compounds (methamphetamine and cocaine), and similar to those of amphoteric and acidic compounds (benzyloleconbine and 11-nortetrahydrocannabinol-9-carboxylic acid). This suggests that acidic and/or amphoteric compounds have a much lower tendency to be incorporated into hair than basic compounds.

**Human Hair Samples**

The developed method was applied to human hair samples. For both PB and PPH, two hair samples were obtained from humans administered the drugs. The PB hair samples were obtained from two emergency patients who took overdoses of Vegetaime A. PB was clearly detected in the hair (see Fig. 7), and the concentrations of PB were determined to be 16.2 and 14.7 ng/mg, respectively. In addition, the \(p\)-hydroxylated metabolite was tentatively detected at trace levels. Hair samples from two volunteers who orally took PPH granules daily for 5 d at the single dose of 100 mg were collected with scissors at the point closest to the skin. The 5 mm portion at the root side was used for analysis. As a result of analysis, PPH was detected in both hair samples and their hair concentrations were 3.3 and 0.1 ng/mg (Fig. 8).

From the overall results, we have highlighted the necessity to choose the appropriate extraction method and analytical method for PB, PPH and their hydroxylated metabolites in hair. Although the ICRs of PB and PPH were considerably low, it is possible to detect and determine them in hair since
AUCs are relatively high. Therefore, our method will be useful for analyzing human hair samples containing PB, PPH and their metabolites for the purpose of practical toxicological analysis.

Acknowledgement

We would like to thank Dr. Makoto Nihira of Nippon Medical School for providing human hair samples.

REFERENCES AND NOTES

1) This work was presented at the Annual Meeting of Pharmaceutical Science of Japan at Gifu in 2000.