ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Simultaneous Rapid Determination Of Fosphenytoin, Phenytoin, And Its Major Metabolite In Human Serum By High-Performance Liquid Chromatography.

Yoko Urashima*, Manami Kiso, Yoshihiko Hirotani, and Kenji Ikeda.

Laboratory of Clinical Pharmaceutics, Faculty of Pharmacy, Osaka Ohtani University, Osaka, Japan.

ABSTRACT

To clarify the pharmacokinetics of FOS by monitoring the conversion of FOS to PHT during treatment, we developed a rapid and simple high-performance liquid chromatographic assay using ultraviolet (UV) detection for the simultaneous determination of FOS, PHT, and its major metabolite 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH). After extracting these compounds and phenobarbital as an internal standard from human serum (50 µL) with acetonitrile, the sample was separated by reverse-phase chromatography on a C_{18} column with a mixture of 10 mM phosphate buffer (pH 7.0):acetonitrile:methanol (160:63:27, v/v) as the mobile phase. The eluent was monitored by UV absorbance at 210 nm. The standard curves of FOS, PHT, and HPPH were linear in the concentration range of 0.63–10 µg/mL. The limit of detection and lower limit of quantification for PHT, FOS, and HPPH were both 0.63µg/mL. The coefficient of variation for replicate samples was less than 10% at each concentration above the limit of quantification for both within- and between-day assay calculations. This method is suitable for analysis of FOS, PHT, and HPPH pharmacokinetics in patients using equipment that is already available and routinely used in many hospitals worldwide.

Keywords: phenytoin, fosphenytoin, 5-(4-hydroxyphenyl)-5-phenylhydantoin, high-performance liquid chromatography, ultraviolet detection, serum.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Parenteral administration of phenytoin (PHT) is widely used for the treatment of acute seizures and status epilepticus, and for the prevention of seizures in epilepsy and neurosurgical patients. Owing to its poor water solubility, PHT is generally dissolved in an alkaline vehicle containing 40% propylene glycol and 10% ethanol, and the solvent can cause adverse events such as injection-site reactions and cardiovascular complications, which pose a challenge for administration planning [1-3]. Fosphenytoin (FOS) is a newly developed prodrug for the parenteral administration of PHT, which improves the low solubility of PHT [4].FOS is converted to PHT by blood and tissue phosphatases with approximately 100% bioavailability[5,6].The greater water solubility alleviates the irritation and phlebitis occurring at the infusion site caused by PHT injection to facilitate the administration of PHT.

However, the pharmacokinetics of FOS in the body remain unclear. One study indicated that the PHT blood concentration did not increase to the therapeutic level in patients who received FOS injection. PHT is extensively para-hydroxylated to its inactive metabolite 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH), which occurs mainly through interaction with the iso-enzymes CYP2C9and CYP2C19 (Fig. 1). To best understand the pharmacokinetics of PHT converted from FOS, it is necessary to determine the specific serum concentrations of PHT, FOS, and HPPH as the indicators of FOS disposition in the patient. Although methods are available for the simultaneous determination of PHT and FOS, or PHT and HPPH[7-9], there is currently no method for the simultaneous determination of PHT, FOS, and HPPH. Individual determination of the FOS, PHT, or HPPH level would reduce the accuracy of the concentration determined for each compound and is time consuming for practical clinical application. Furthermore, a method for the simultaneous determination of PHT, FOS, and HPPH should be available in the hospital to measure patient serum samples. These markers can be determined using high-performance liquid chromatography (HPLC)and a general detector, which are already widely used in many medical institutions. Thus, a simple and fast one-step detection method in the same serum sample using HPLC and ultraviolet (UV) detection is desired. Moreover, this assay could help monitor patients receiving FOS injection with a low serum PHT concentration, which is routinely determined by an immunoassay method. In these cases, the lower limit of quantification (LLOQ) for PHT must be around 0.5 µg/mL because the current LLOQ of the immunoassay is 0.5 µg/mL.

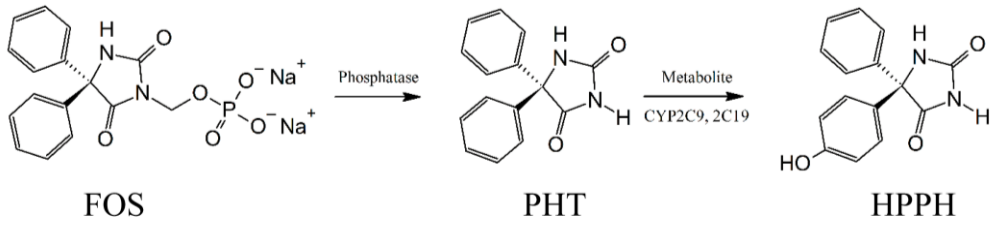

Fig 1: In vivo conversion of fosphenytoin (FOS) to phenytoin (PHT), and metabolism of PHT to 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH).

To resolve these issues, we here present an HPLC assay with UV detection for the simultaneous determination of FOS, PHT, and HPPH in human serum. The method is simple, fast, and applicable for both patient monitoring and pharmacokinetics analyses in many hospitals.

EXPERIMENTAL

MATERIALS

All chemicals were of analytical-reagent grade. PHT (5-5-diphenylhydantoin), HPPH (5-(4-hydroxyphenyl)-4-phenylhydantion), and phenobarbital as the internal standard were purchased from Sigma-Aldrich Japan. Fostoin® 750 mg was used as the FOS for injection, which was purchased from Nobel Pharma Co., Ltd. Distilled water was obtained from Nacalai Tesque Co., Ltd. Pooled human serum (LDT: 14070422) was obtained from Cosmo Bio Co., Ltd.

Standard solutions

PHT and HPPH solutions were prepared in methanol (100 µg/mL), and FOS was dissolved in distilled water (100 µg/mL). The solutions were stored at−20°C and further diluted to appropriate concentrations with phosphate buffer. The internal standard was prepared by resolving in acetonitrile (10 µg/mL) and stored at 4°C until use.

Extraction procedure

To prepare the solution for constructing the calibration curve, the FOS, PHT, and HPPH solutions were added to 200 µL of pooled human serum to prepare solutions containing 0.63 to10.0 µg/mL of each chemical; 50 µL of the internal standard was added to 50 µL of the calibration solution. The solution was vortexed for 30 sec and centrifuged at 10,000 rpm for 20 min at 4°C. Fifty microliters of the supernatant was transferred to a clean tube, and 20 µL was injected into the HPLC system.

Instrumentation

The Simadzu VP-series HPLC system was used in this study, consisting of an LC-20AD pump, DGU-20A deaeration unit, CTO-20Avp column oven, SPD-20ADvp UV detector, and SIL-20ADvp auto-injector, controlled by an SCL-10 Avp controller (Shimadzu Co. Japan).

Chromatographic conditions

Separations were performed on a Cosmosil® $5C_{18}$-PAQ column (4.6 mm I.D.×150 mm, Nacalai Tesque Co., Ltd.), preceded by a run through the Cosmosil® $5C_{18}$-MS-II guard column (4.6 mm I.D.×10 mm).In a preliminary assay, we tested different HPLC conditions of the mobile phase ratio[10 mM phosphate buffer (pH 7.0): acetonitrile and methanol=66:54 or 70:50, and acetonitrile:methanol= 2:8 or 7:3 (v/v)] and the total flow rate (1.0 or 1.2 mL/min). Based on the results, we determined the optimal conditions to be a mobile phase of 10 mM phosphate buffer (pH 7.0):acetonitrile:methanol(160:63:27 (v/v)), and a flowrate of 1.0 mL/min. The column temperature was maintained at 35°C to assure reproducibility of the retention time. Eluting peaks were monitored by UV absorbance at 210 nm.

Linearity

Each calibration curve was created using five points (0.63, 1.25, 2.5, 5.0, and 10.0 µg/mL) of FOS, PHT, and HPPH in human pooled serum. Linear regression was used to fit peak-area ratio-plasma concentration data.

Precision

The precision of the method was determined in relation to repeatability (intra-day and between-day).To evaluate the repeatability of this method, five mixture solutions of each FOS, PHT, and HPPH concentration were prepared independently from the calibrators, and analyzed on the same day or on separate days. The final results are reported as the coefficient of variation (CV) of the FOS, PHT, and HPPH peak area ratios.

Accuracy

The accuracy of the method was calculated as the percentage recovery: $x_A/\mu \times 100\%$, where x_A is the analyzed amount of either FOS, PHT, or HPPH in the sample, and µ is the known amount of the substance in the sample.

ISSN: 0975-8585

Recovery rate

The recovery rate (%) of the drug from plasma was determined by comparing extracted samples containing known amounts FOS, PHT, and HPPH from human pooled serum, with samples directly prepared in acetonitrile and not extracted.

RESULTS AND DISCUSSION

HPLC conditions and chromatogram

After extensive preliminary experimental trials, baseline separation of FOS, PHT, HPPH, and the internal standard was achieved with symmetrical peaks obtained in less than 12 min when using an isocratic elution with a mobile phase consisting of 10 mM phosphate buffer:acetonitrile:methanol =160:63:27 (pH7.0) at a flow rate of 1.0 mL/min. Fig. 2 shows a chromatogram of the standard mixture of FOS, PHT, HPPH, and internal standard with retention times of 3.8 min, 9.5 min, 4.8 min, and 5.3 min, respectively. There was no interference detected at the drug and internal standard peaks. Separation and detection of FOS, PHT, HPPH, and the internal standard were completed promptly.

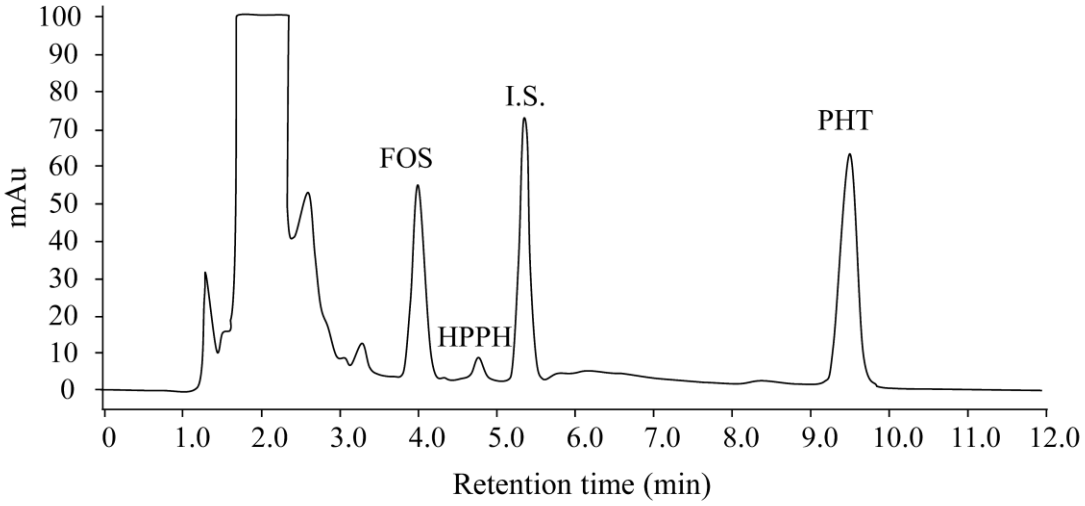

Fig 2: Chromatograms of an extract of a standard sample with fosphenytoin (FOS), phenytoin (PHT), and 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH) in human serum. FOS and PHT concentration, 10 µg/mL; HPPH concentration, 0.625 µg/mL; internal standard (I.S.) concentration, 5 µg/mL.

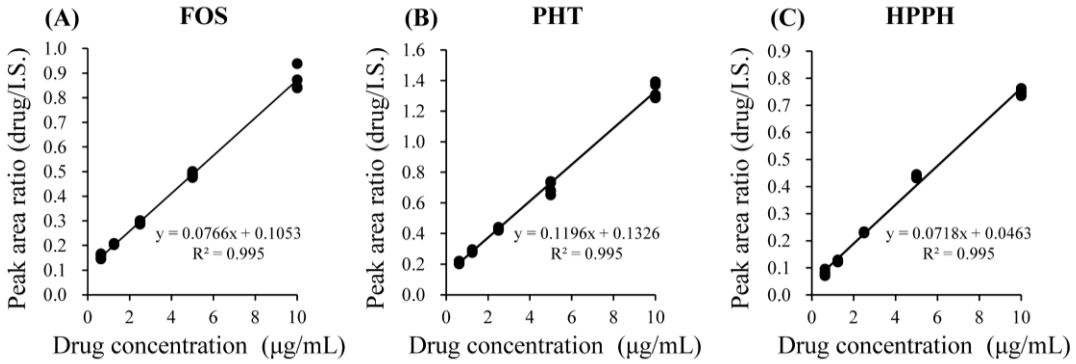

Fig 3: Calibration curve for (A) fosphenytoin (FOS), (B) phenytoin (PHT), and (C) 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH) based on the linear relationship between the ratio of the peak area of the standard to that of the internal standard (I.S., y-axis) and the concentration of the standard (x-axis). Data are shown as the mean ±S.D. (n = 5)

ISSN: 0975-8585

Method validation

The calibration curves for FOS, PHT, and HPPH were obtained using a series of standard solutions (Fig. 3).A linear relationship between the ratio of the peak area of the standard to that of the internal standard (y) and the concentration of the standard (x) was used. The regression equations (correlation coefficient)for FOS, PHT, and HPPH demonstrated favorable linearity in all cases.

The limit of detection and LLOQ for FOS, PHT, and HPPH were both 0.63 µg/mL. FOS LLOQ demonstrated comparable sensitivity with that reported in a previous study using HPLC with UV detection(0.403 µg/mL)[7], and the PHT LLOQ matched with that of the currently used PHT immunoassay (0.5 µg/mL).

The within- and between-day precisions were examined by performing the analyses five times. As shown in Table 1, the relative CV did not exceed 10% in any of the cases tested, demonstrating good reliability and reproducibility of the assay.

Table 1: Within- and between-day precisions and accuracy of fosphenytoin (FOS), phenytoin (PHT), and 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH) detection

Concentration	Within-day			Between-day		
(µg/mL)	Mean ± S.D.	CV (%)	Accuracy(%)	Mean ± S.D.	CV (%)	Accuracy(%)
FOS						
0.63	0.60± 0.04	1.9	95.2	0.61 ± 0.03	1.6	96.9
1.25	1.22± 0.09	3.3	97.4	1.14 ± 0.13	5.0	91.5
2.50	2.51± 0.17	4.4	100.5	2.48 ± 0.32	8.3	99.2
5.00	4.71± 0.25	4.1	94.2	4.57 ± 0.32	5.3	91.4
10.00	8.85± 0.21	2.1	88.5	8.83 ± 0.72	7.1	88.3
PHT						
0.63	0.60± 0.03	2.0	96.3	0.64 ± 0.06	3.2	103.0
1.25	1.16± 0.14	6.1	93.0	1.23 ± 0.16	6.7	98.6
2.50	2.32± 0.13	3.9	93.0	2.50 ± 0.35	9.6	99.8
5.00	5.00± 0.22	3.7	100.0	4.89 ± 0.35	5.8	97.8
10.00	10.59± 0.38	3.2	105.9	10.34 ± 0.67	5.9	103.4
HPPH						
0.63	0.65± 0.04	6.1	104.8	0.62 ± 0.05	8.1	98.8
1.25	1.24± 0.02	1.3	99.4	1.28 ± 0.06	4.9	102.1
2.50	2.62± 0.06	2.2	104.8	2.72 ± 0.15	5.4	108.6
5.00	4.52± 0.14	3.1	90.3	4.48 ± 0.19	4.2	89.5
10.00	10.69± 0.79	7.4	106.9	10.96 ± 0.90	8.2	109.6

CV, Coefficient of variation. Data are shown as the mean ±S.D. (n = 5)

Extraction performance

The recovery rates of FOS, PHT, and HPPH are shown in Table 2, demonstrating excellent recovery of PHT. Although the recovery for FOS and HPPH was lower than that of PHT, the standard deviations for each compound were very small and still showed high availability. A previous report indicated a requirement of a 100 µL serum sample along with evaporation and reconstitution procedures for preparation of serum samples for analysis of PHT and FOS [7]. Thus, one of the major advantages of the present method is that FOS, PHT, and HPPH can be determined with only a 50 µL serum sample in a single analysis, and the extraction procedure is very simple without requiring evaporation and reconstitution steps.

Furthermore, this extraction method takes only approximately 30 min until measurement, making it highly practical for clinical use in therapeutic drug monitoring.

Table 2: Recovery rates of fosphenytoin (FOS), phenytoin (PHT), and 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH)

Concentration (μg/mL)	Recovery rate (%)		
FOS			
0.63	70.6	±	2.7
1.25	65.7	±	3.5
2.50	61.5	±	2.6
5.00	75.2	±	3.0
10.00	77.6	±	2.9
PHT			
0.63	95.4	±	3.5
1.25	102.5	±	3.9
2.50	97.6	±	4.6
5.00	104.5	±	5.7
10.00	92.4	±	3.1
HPPH			
0.63	64.5	±	2.0
1.25	60.2	±	3.0
2.50	60.2	±	2.4
5.00	66.1	±	2.3
10.00	71.9	±	2.0

Data are shown as the mean ± SD (n = 5)

CONCLUSION

To achieve fast and convenient measurements of accurate FOS, PHT, and HPPH serum concentrations in patients, development of a simultaneous detection assay is in high clinical demand. In this study, we developed the first method using HPLC and UV detection for the simultaneous determination of FOS, PHT, and HPPH in human serum, which requires only 30 min from pre-treatment of the serum sample to detection, without requiring an evaporation and reconstitution procedure. In addition, since this equipment is available in most medical institution, it can be widely applied without requiring additional costs or training. This rapid and simple method is suitable and immediately available for analyses of FOS, PHT and HPPH pharmacokinetics in patients with a low blood PHT concentration after receiving FOS injection.

Competing Interests: None declared.

REFERENCES

[1] Sale RB, Wilder BJ, Yost RL, Doering PL, Lee C. Rapid infusion of phenytoin sodium loading doses. Am J Hosp Pharmacol 1981;38:354-357.

[2] Spengler RF, Arrowsmith JB, Kilarski DJ, Buchanan C, Von Behren L, Graham DR. Severe soft-tissue injury following intravenous infusion of phenytoin. Patient and drug administration risk factors. Arch Intern Med 1988;148: 1329-1333.

[3] Louis S, Kutt H, McDowell F. The cardiocirculatory changes caused by intravenous Dilantin and its solvent. Am Heart J 1967;74:523-529.

[4] Leppik IE, Boucher BA, Wilder BJ, Murthy VS, Watridge C, Graves NM, Rangel RJ, Rask CA, Turlapaty P. Pharmacokinetics and safety of a phenytoin prodrug given i.v. or i.m. in patients. Neurology 1990;40:456-460.

[5] Boucher BA, Bombassaro AM, Rasmussen SN, Watridge CB, Achari R, Turlapaty P. Phenytoin prodrug 3-phosphoryloxymethyl phenytoin (ACC-9653): pharmacokinetics in patients following intravenous and intramuscular administration. J Pharm Sci 1989;78:929-932.

[6] Jamerson BD, Donn KH, Dukes GE, Messenheimer JA, Brouwer KL, Powell JR. Absolute bioavailability of phenytoin after 3-phosphoryloxymethyl phenytoin disodium (ACC-9653) administration to humans.

Epilepsia 1990;31:592-597.

[7] Cwik MJ, Liang M, Deyo K, Andrews C, Fischer J. Simultaneous rapid high-performance liquid chromatographicdetermination of phenytoin and its prodrug, fosphenytoin inhuman serum and ultrafiltrate. J Chromatogr B Biomed 1997;693:407-414.

[8] Ferreira1 A, Rodrigues M, Falcão A, Alves G. A rapid and sensitive HPLC–DAD assay toquantify lamotrigine, phenytoin and its mainMetabolite in samples of cultured HepaRG cells.J Chromatogr Sci2016;54:1352-1358.

[9] Hara S, Hagiwara J, Fukuzawa M, Ono N, Kuroda T. Determination of phenytoin and its major metabolites in human serum by high-performance liquid chromatography with fluorescence detection. Anal Sci 1999;15:371-375.