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Simultaneous separation and determination (in serum) of phenytoin and carbamazepine and their deuterated analogues by high-performance liquid chromatography–ultraviolet detection for tracer studies

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ABSTRACT

The use of stable isotope-labeled tracer compounds is the safest and most effective method to perform many steady state pharmacokinetic and drug interaction studies. We describe a method by which the heavily deuterated $^{2}H_{10}$ analogues of carbamazepine ($^{2}H_{10}$ CBZ) and phenytoin ($^{2}H_{10}$ PHT) can be chromatographically separated by high-performance liquid chromatography from unlabeled CBZ and PHT. All compounds are quantitated against an internal standard (IS) (10,11-dihydrocarbamazepine) and measured using conventional UV detection rather than mass spectrometry. Baseline resolution of extracted serum containing $^{2}H_{10}$ CBZ, CBZ, $^{2}H_{10}$ PHT, PHT and IS is achieved on a heated (55°C) 25 cm × 4.6 mm BioAnalytical Systems Phase II 5 μm ODS column with an isocratic mobile phase consisting of water–acetonitrile–tetrahydrofuran (80:16:4, v/v/v) at 1.2 ml/min. Eluting compounds were monitored at a UV wavelength of 214 nm. Calculated resolution of $^{2}H_{10}$ CBZ from CBZ and of $^{2}H_{10}$ PHT from PHT were 1.3. Serum standard curves were linear ($R \geq 0.999$) over a range of 0.5–14 μg/ml for $^{2}H_{10}$ CBZ, 0.5–20 μg/ml for CBZ, 0.5–20 μg/ml for $^{2}H_{10}$ PHT, and 0.5–30 μg/ml for PHT. Within-day percent relative standard deviations (precision) were less than 6% in all cases.

INTRODUCTION

Stable isotope-labeled (SIL) tracer methods have many demonstrated advantages over alternative methods in human pharmacokinetic and drug interaction studies [1,2]. Nevertheless, SIL tracer methods are not widely used [3], largely because of the cost and scarcity of gas chromatographic–mass spectrometry (GC–MS) equipment. Separation of a drug and its heavily deuterated analogue by capillary GC

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[4,5] and also by high-performance liquid chromatography (HPLC) [6] has been described; however, the potential of this technique has not been exploited in human tracer studies. We report a method for quantitating the concentration of unlabeled and SIL carbamazepine (CBZ) and phenytoin (PHT) in serum suitable for tracer studies using only a simple isocratic HPLC system with conventional ultraviolet (UV) detection.

EXPERIMENTAL

Instrumentation

This study employed a Waters Model 6000A solvent pump and a Model 710B WISP autosampler (Waters Assoc., Milford, MA, U.S.A.). Eluents were monitored using a Spectroflow Model SF770S variable-wavelength detector (Applied Biosystems, Ramsey, NJ, U.S.A.). Column temperature was maintained by a Model LC22A column heater (BioAnalytical Systems, West Lafayette, IN, U.S.A.). Chromatograms were recorded on a Model DB111 flatbed recorder (Kipp and Zonen, Delft, The Netherlands) and also on a Packard Bell PBVX88 computer for peak integration and quantitation using GrayMatter™ software (Binary Systems, Newton, MA, U.S.A.).

Standards and reagents

The internal standard, 10,11-dihydrocarbamazepine and 5,5-diphenylhydantoin (phenytoin) were obtained from Aldrich (Milwaukee, WI, U.S.A.). The 5H-dibenz[*b*,*f*]azepine-5-carboxamide (carbamazepine) was supplied by Sigma (St. Louis,

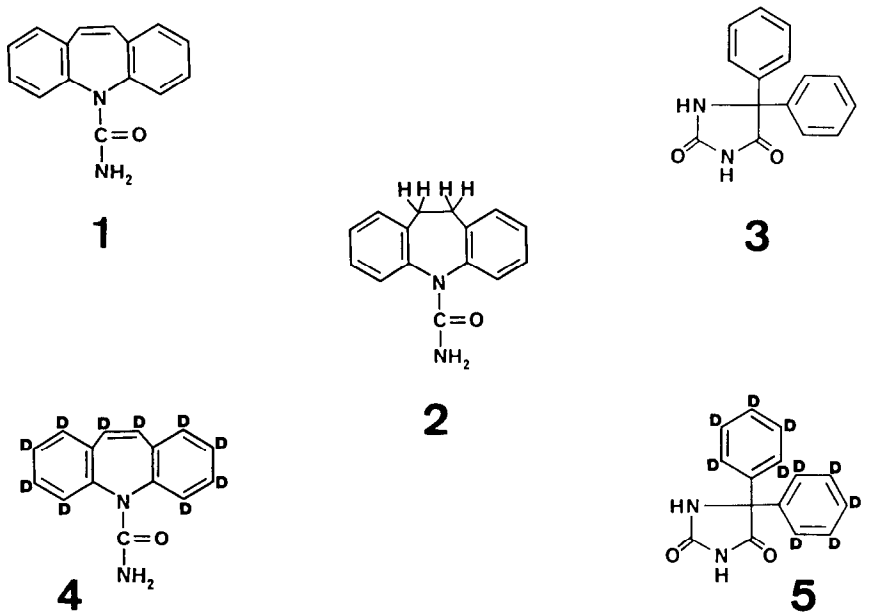

Fig. 1. Chemical structures and isotopic labeling of standards. 1 = CBZ; 2 = 10,11-dihydro-CBZ (internal standard); 3 = PHT; 4 = [$^2H_{10}$]CBZ; 5 = [$^2H_{10}$]PHT; D = deuterium.

MO, U.S.A.) and the [$^2H_{10}$]CBZ was from MSD Isotopes (Montreal, Canada). The [$^2H_{10}$]PHT was obtained from Tracer Technologies (Somerville, MA, U.S.A.). Chemical structures and isotopic labeling sites are shown in Fig. 1. HPLC-grade ethyl acetate, methylene chloride, acetonitrile, tetrahydrofuran (THF), and methanol were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Monobasic and dibasic potassium phosphate were obtained from Mallinckrodt (Paris, KY, U.S.A.).

Phosphate buffer (pH 6.0 $\pm$ 0.05) solution was prepared by addition of dibasic potassium phosphate (0.067 mol/l) to 750 ml of monobasic potassium phosphate (0.067 mol/l) with stirring while monitoring the pH potentiometrically. Stock analytical and internal standard solutions for CBZ, [$^2H_{10}$]CBZ, PHT, [$^2H_{10}$]PHT and 10,11-dihydro-CBZ were prepared by dissolving 10 mg of each compound in methanol and diluting to 10 ml. Methanolic working standards containing all analytes at varying concentrations for calibration and controls were prepared by dilution of stock solutions. Working internal standard solution was prepared in methanol–distilled water (1:1) at a concentration of 50 μg/ml.

Chromatographic conditions

The mobile phase consisted of HPLC-grade water–acetonitrile–THF (80:16:4, v/v/v). The separation was achieved isocratically on a BioAnalytical Systems Phase II ODS 5-μm (25 cm $\times$ 4.6 mm I.D.) stainless-steel column that was maintained at 55°C. The flow-rate was 1.2 ml/min and eluent was monitored at 214 nm with a sensitivity of 0.05 a.u.f.s.

Sample preparation

Calibrants and control samples were prepared by evaporating 1.0 ml of the various working standard solutions in 125 mm $\times$ 16 mm disposable glass culture tubes and adding 1.0 ml of drug free serum. To 1.0 ml of control or patient serum 1.0 ml of phosphate buffer (pH 6.0) and 100 μl of working internal standard were added. The tubes were swirled briefly and then extracted by adding 8.0 ml ethyl acetate and 1.0 ml methylene chloride. Samples were capped with polyethylene Tainer Tops (Fisher Scientific) and set on a horizontal shaker (Eberbach Corp., Ann Arbor, MI, U.S.A.) for 10 min at 180 cycles/min, then centrifuged at room temperature long enough to break any emulsion that may have formed. The samples were then immersed in a methanol–dry ice bath for 15 min to freeze the aqueous (lower) layer. A subsequent brief (30–45 s) centrifugation (2000 rpm) at -10°C settled any remaining frozen aqueous crystals suspended in the organic layer. Then the organic layer was decanted into a clean tube for concentration and placed on a Meyer N-VAP evaporator (Organomation, Berlin, MA, U.S.A.). After evaporation of the organic fraction under a gentle stream of air at 45°C, the residue was reconstituted with 200 μl of methanol, vortexed, and transferred to WISP autosampler vials. At this point, 15–30 μl of sample was injected on the HPLC.

RESULTS

Precision and accuracy

Each working methanolic standard had combined in it CBZ (0.5–20.0 μg/ml), [$^2H_{10}$]CBZ (0.5–14.0 μg/ml), PHT (0.5–30.0 μg/ml), and [$^2H_{10}$]PHT (0.5–20.0 μg/ml).

Three 13-point calibration curves were prepared from methanolic standard solutions and drug-free serum for determination of linearity, and within-day accuracy and precision. All samples were prepared according to the described method. Unweighted linear least squares regression analysis of peak height ratio *versus* analyte concentration was performed, and residuals were calculated. Correlation coefficients (linearity) for all four analytes were equal to or greater than 0.999. Percent relative standard deviations (precision) were less than 6% in all cases. Fig. 2 shows the calibration plots with residuals (accuracy) calculated using the method of Johnson *et al.* [7] shown as percentages for all analytes. Residuals were within 8% for CBZ and $[^2H_{10}]$CBZ, and within 4% for all concentrations greater than 1 μg/ml. The lowest concentrations of PHT and $[^2H_{10}]$PHT contributed to high negative residuals; however, all concentrations above 0.5 μg/ml had residuals less than 8%. A weighted regression analysis would probably decrease the residuals at low concentrations.

Fig. 3 shows typical chromatograms obtained under the specified experimental conditions.

DISCUSSION

Sample preparation

The freezing out extraction technique employed in this method was a modifica-

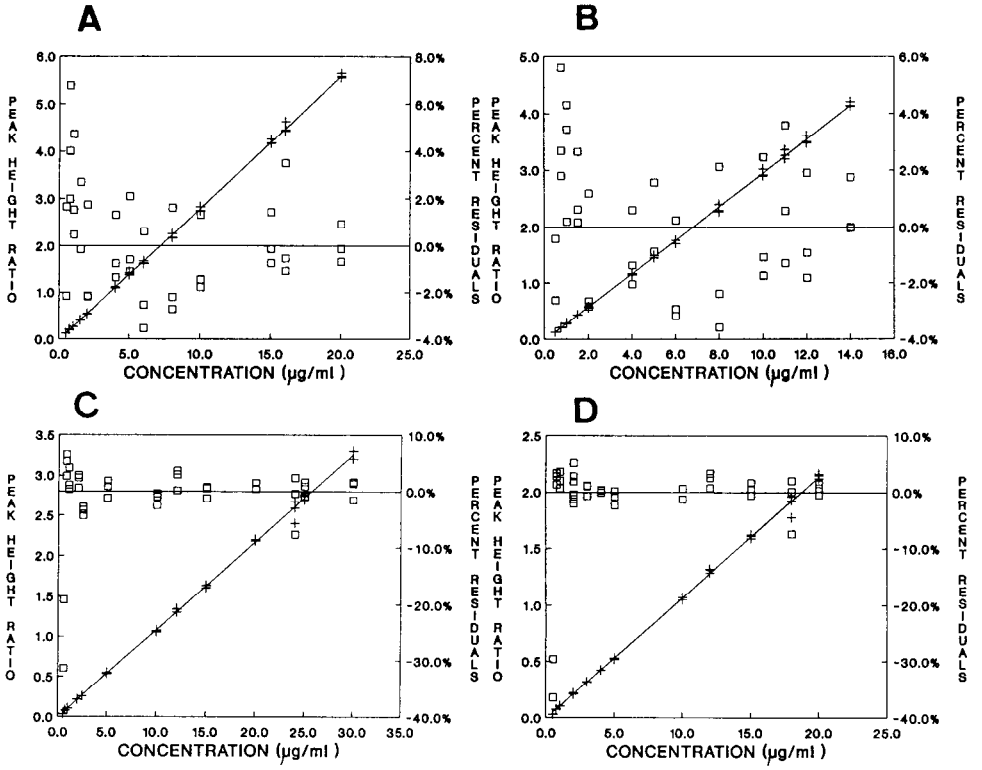

Fig. 2. Calibration plot with residuals (%) for CBZ (A), $[^2H_{10}]$CBZ (B), PHT (C) and $[^2H_{10}]$PHT (D). + = Peak height ratio; □ = residuals; ——— = regression.

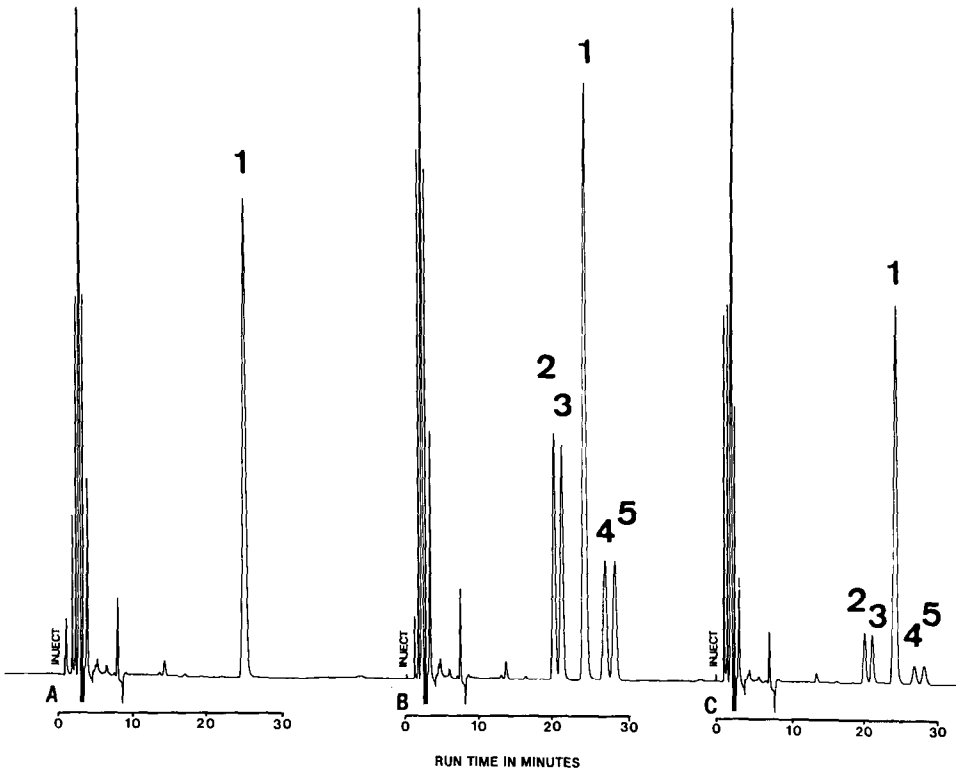

Fig. 3. Chromatograms of 15-μl injections of: blank serum containing 10,11-dihydro-CBZ (internal standard) 5.0 μg/ml (A); serum control containing [$^2H_{10}$]CBZ, CBZ 1.5 μg/ml and [$^2H_{10}$]PHT, PHT 2.0 μg/ml (B); serum calibrant containing [$^2H_{10}$]CBZ, CBZ, [$^2H_{10}$]PHT and PHT 0.5 μg/ml (C). Peaks: 1 = 10,11-di-hydro-CBZ; 2 = [$^2H_{10}$]CBZ; 3 = CBZ; 4 = [$^2H_{10}$]PHT; 5 = PHT.

tion of a procedure described by Schmidt *et al.* [8]. Their method was a multiple step hexane extraction, back extraction and re-extraction performed in a batch mode with time to freeze samples between steps in a $-20°C$ freezer. A freezer is also applicable with this procedure; however, flash freezing in the dry ice bath is faster. The quick $-10°C$ centrifugation step was necessary because, unlike hexane, ethyl acetate absorbs water which freezes out of solution and becomes ice crystals suspended in the organic layer. After centrifugation the dried organic fraction was quickly and easily poured off into the evaporation tube. Besides the ease and speed of not having to transfer the aqueous layer by pipetting, fast evaporation was facilitated by the removal of absorbed water.

Chromatography

On most C_{18} reversed-phase columns used for the separation of CBZ and PHT, a binary mobile phase of acetonitrile and water gives an elution order in which CBZ follows PHT. The addition of a small percentage of THF reversed the elution order since THF has a much greater effect on the retention of CBZ, and seems also to facilitate the separation of drug from its isotopomer (Fig. 3). The use of a heated

column is necessary to prevent run times from becoming too lengthy for practical applications. Total run times under the conditions described here varied from 28 to 32 min. Even though these run times seem long compared to conventional clinical HPLC assays, the ease of sample preparation (without derivatization, etc.) and use of automation makes this method a viable alternative to GC–MS procedures. Chromatographic resolution (R_s) between drugs and their isotopomers in serum controls was 1.3. Resolution [9] was defined as:

$$R_s = \frac{t_2 - t_1}{0.5(w_1 + w_2)}$$

where t_1 and t_2 are retention times of adjacent components ($t_2 > t_1$), and w_1 and w_2 are base widths of the peaks in time units.

Deuterium is the most cost-effective stable isotope to use for SIL labeling in terms of synthesis and market availability. Depending on placement of the deuterons in the drug molecule, *in vivo* or *in vitro* "isotope effects" can be negligible or profound [10–14]. Tanaka and Thornton [15] first described a mechanism for liquid chromatographic separation of labeled and unlabeled compounds based on hydrophobic interactions. It was also shown that isotope effect changes proportionately with the number of deuterons used [16]. Another way to characterize the isotope effect is as a reduction of lipophilicity [17]. As an index of lipophilicity, strong correlations have been shown between changes in partition coefficients and chromatographic retention of heavily deuterated aromatic compounds [17–19] and drugs with aromatic rings in their structures [19,20]. Absence of isotope effects of [$^2H_{10}$]PHT to PHT has been demonstrated in human pharmacokinetic studies [18–20].

CONCLUSIONS

The use of SIL compounds in pharmaceutical research has not gained universal acceptance, in part because of the cost and technical complexities associated with analytical MS. This paper describes an assay procedure for the simultaneous separation and quantitation of the two most commonly used antiepileptic drugs and their decadeuterated analogues [$^2H_{10}$]CBZ, [$^2H_{10}$]PHT) for SIL dilution studies. The freeze extraction procedure described allows for simple, rapid sample preparation and offers both a time and a cost effective alternative to mass spectrometry. Tremendous advances have been made in particle and surface chemistries yielding highly selective and efficient HPLC columns that could be used for assay of a variety of heavily deuterated pharmaceuticals. Assuming no pharmacokinetically deleterious *in vivo* isotope effect for a heavily deuterated compound is demonstrated, SIL tracer studies for pharmaceutical development, drug interaction studies and pharmacokinetic research could be conducted using HPLC without MS detection. A study of possible pharmacokinetic isotope effects of [$^2H_{10}$]CBZ and CBZ is underway. This paper is a preliminary study demonstrating the capabilities of this assay. Additional validation studies are in progress to fully characterize this method (between-day precision, absolute recovery, and limits of minimum detection).

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REFERENCES

1 T. R. Browne, *Clin. Pharm.*, 18 (1990) 423.
2 T. R. Browne, D. J. Greenblatt, G. E. Schumacher, G. K. Szabo, J. E. Evans, B. A. Evans, R. J. Perchalski and R. J. Pylilo, in W. H. Pitlick, H. J. Kupferberg, R. H. Levy, R. J. Porter and R. H. Mattson (Editors), *Antiepileptic Drug Interactions*, Demos, New York, 1972, p. 3.
3 R. L. Wolen, in T. A. Baillie and J. R. Jones (Editors), *Synthesis and Applications of Isotopically Labeled Compounds, Proc. 3rd Int. Symp., Innsbruck, July 17–21, 1988*, Elsevier, Amsterdam, 1989, pp. 147–156.
4 D. J. Hoffman and W. R. Porter, *J. Chromatogr.*, 276 (1983) 301.
5 W. A. Van Hook, *J. Chromatogr.*, 338 (1985) 333.
6 T. K. Gerding, B. F. H. Drenth and R. A. de Zeeuw, *Anal. Biochem.*, 171 (1988) 382.
7 E. L. Johnson, D. L. Reynolds, D. S. Wright and L. A. Pachla, *J. Chromatogr. Sci.*, 26 (1988) 372.
8 J. Schmid and F.-W. Koss, *J. Chromatogr.*, 227 (1982) 71.
9 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979.
10 M. I. Blake, H. L. Crespi and J. J. Katz, *J. Pharm. Sci.*, 64 (1975) 367.
11 A. Van Langenhove, *J. Clin. Pharm.*, 26 (1986) 383.
12 S. Ottoboni, P. Caldera, A. Trevor and N. Castagnoli, Jr., *J. Biol. Chem.*, 264 (1989) 13684.
13 J. P. Thenot, T. I. Ruo, G. P. Stec and A. J. Atkinson, in A. Frigerio and M. McCamish (Editors), *Recent Developments in Biochemistry and Medicine, 6*, Elsevier, Amsterdam, 1980, p. 373.
14 Y. Cherrah, J. B. Falconnet, M. Desage, J. L. Brazier, R. Zini and J. P. Tillement, *Biomed. Env. Mass Spectrom.*, 14 (1987) 653.
15 N. Tanaka and E. R. Thornton, *J. Am. Chem. Soc.*, 98 (1976) 1617.
16 N. Tanaka and E. R. Thornton, *J. Am. Chem. Soc.*, 99 (1977) 7300.
17 N. El Tayar, H. van de Waterbeemd, M. Gryllaki, B. Testa and W. F. Trager, *Int. J. Pharm.*, 19 (1984) 271.
18 I. M. Kovach and D. M. Quinn, *J. Am. Chem. Soc.*, 105 (1983) 1947.
19 G. P. Cartoni and I. Ferreti, *J. Chromatogr.*, 122 (1976) 287.
20 J. B. Falconnet, N. El Tayar, A. Bechalany, Y. Cherrah, V. Benchekroun, P. A. Carrupt, B. Testa and J. L. Brazier, in T. A. Baillie and J. R. Jones (Editors), *Synthesis and Applications of Isotopically Labeled Compounds, Proc. 3rd Int. Symp. Innsbruck, July 17–21, 1988*, Elsevier, Amsterdam, 1989, pp. 355–360.
21 Y. Kasuya, K. Mamada, S. Baba and M. Matsukura, *J. Pharm. Sci.*, 74 (1985) 503.
22 K. Kamada, Y. Kasuya and S. Baba, *Drug Met. Disp.*, 14 (1986) 509.
23 M. Claesen, M. Moustafa, J. Adline, D. Vandervorst and J. Poupaert, *Drug Met. Disp.*, 10 (1982) 667.