Journal of Chromatography, 374 (1986) 170—176
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2827

Note

Resolution of verapamil from heptadeuteroverapamil and their quantitation in serum using capillary gas chromatography

D.J. HOFFMAN* and J. HIGGINS

Pharmaceutical Products Division, D-493, Abbott Laboratories, North Chicago, IL 60064 (U.S.A.)

(First received April 10th, 1985; revised manuscript received August 9th, 1985)

Verapamil is a calcium-channel blocking vasodilator primarily indicated for anginas and arrhythmias. It is almost completely absorbed after oral administration but the absolute bioavailability is low (20%) owing to extensive hepatic first-pass metabolism [1]. Often with drugs that have a substantial first-pass effect, large inter-individual and day-to-day intra-individual variations occur. These variations cause problems with conventional, relative bioavailability studies which assume a relatively invariant metabolic state [2]. For this reason, stable-isotope techniques have been recommended for verapamil [3]. Until recently, the only technique available to determine both a light- and heavy-isotope-labeled drug in the same sample was gas chromatography—mass spectrometry (GC—MS). However, a gas chromatograph—mass spectrometer is often not readily available for the routine analysis of a large number of biological samples. An option to GC—MS is to quantitate deuterated compounds using capillary GC. Hoffman and Porter [4] recently demonstrated that capillary GC is capable of resolving and quantitating deuterated valproic acid analogues from valproic acid. As demonstrated by Berger et al. [5] the method is not limited to deuterated compounds. They used capillary GC to separate carbon and hydrogen isotopes of methane. In this paper, we present a capillary GC method in combination with nitrogen-specific detection which is capable of quantitating verapamil and a deuterated analogue in human serum.

0378-4347/86/$03.50

EXPERIMENTAL

Chemicals and reagents

Verapamil hydrochloride was U.S.P. d_7-Verapamil was synthesized at Abbott Labs. starting with d_7-isopropyl bromide and dimethoxyphenylacetonitrile (Aldrich, Milwaukee, WI, U.S.A.) [6]. d_7-Isopropyl bromide was purchased from MSD Isotopes (Dowal, Canada). Internal standard, the *n*-propyl analogue of verapamil, was prepared by the route described for d_7-verapamil using *n*-propyl bromide (Aldrich). Assigned structures were in agreement with their respective NMR, IR and mass spectra.

Stock solutions of verapamil and d_7-verapamil were prepared in ethanol at concentrations of about 1 mg/ml. Secondary stock standard solutions in 0.01 *M* hydrochloric acid containing both verapamil and d_7-verapamil in weight ratios of 1:2, 1:1 and 2:1 were prepared at a concentration of 100 μg/ml of the dominant drug. For example, a verapamil/d_7-verapamil ratio of 2:1 results in a secondary stock standard containing verapamil at 100 μg/ml and d_7-verapamil at 50 μg/ml. A series of working standard solutions in 0.01 *M* hydrochloric acid was prepared from dilutions of an initial working standard at 2.4 μg/ml (6 ml of secondary standard diluted to 250 ml). The series of working solutions were prepared so that 1.0 ml of each working standard when added to a 25-ml volumetric flask and adjusted to volume with serum resulted in serum concentrations ranging from 2 to 100 ng/ml. Internal standard solution was prepared at about 0.4 μg/ml in 0.0025 *M* sulfuric acid.

All other chemicals and reagents were of analytical-reagent grade.

Gas chromatography

A fused-silica WCOT column (60 m × 0.25 mm I.D.) with an SE-52 bonded liquid phase of 0.1 μm thickness (DB-5, J&W Scientific, Rancho Cordova, CA, U.S.A.) was used with a Hewlett-Packard Model 5840A gas chromatograph equipped with an alkali flame-ionization detector and autosampler. Because of the small reconstituted sample volume (70 μl) the normal sampling sequence, which pre-rinses the injection syringe with 50 μl of sample, could not be used. An optional PC board (Part No. 18824-60101, Hewlett-Packard) was used which removed only the sample volume (3 μl) from the vial. The syringe was rinsed, after each sample vial, with alternate vials containing 0.01% triethylamine in ethanol. The injection port was maintained at 280°C. A 78 mm × 2.3 mm I.D. borosilicate glass insert was used. To maintain proper sample recovery and chromatographic efficiency the insert was cleaned about every 100 samples by heating it just to boiling in 100 ml of 5 *M* sodium hydroxide. The insert was rinsed with 20 ml of distilled water followed by 20 ml of acetone. Samples were injected automatically in the splitless mode with a purge activation time of 0.7 min. The hydrogen carrier gas flow-rate was adjusted to give a partition ratio (k) of 5—6 for verapamil. This corresponds to linear flow-rates of 25—30 cm/s. The initial oven temperature was 185°C. After 0.65 min at 185°C the oven temperature was programmed at 25°C/min until it reached 275°C where it was held for about 17 min. The detector temperature was 290°C, with hydrogen, helium (make-up gas), and air flow-rates of 2.7, 40 and 60 ml/min, respectively. The chart speed was 0.1

cm/min. To aid in the visualization of the peaks the chart speed was automatically increased to 1 cm/min just prior to the elution of the verapamil peaks.

Assay procedure

A 1-ml C_{18} reversed-phase extraction column (J.T. Baker, Phillipsburg, NJ, U.S.A.) was rinsed with one column volume of methanol followed by one volume of distilled water. To a disposable glass test-tube (75 mm × 10 mm) were added 0.5 ml of internal standard solution, 1 ml serum sample or standard and 0.2 ml of 0.5 *M* sodium carbonate. The sample was mixed by vortexing and passed through the extraction column at a flow-rate less than 2 ml/min. The extraction column was successively washed with one volume of distilled water and 0.5 ml of 50% methanol in water. Verapamil and the internal standard were eluted from the column with one volume of ethanol containing 0.1% triethylamine. The eluent was evaporated to dryness at 35°C with a gentle stream of dry filtered air. The residue was reconstituted with 70 μl of ethanol containing 0.1% triethylamine, mixed by vortexing, and transferred to an autosampler microvial and capped. After tightly capping the microvial, a 3-μl aliquot was automatically injected into the gas chromatograph. All samples and standards were randomly injected.

Serum standards were prepared in unlabeled/labeled ratios of 1:2, 1:1 and 2:1. A calibration curve slope was determined for each ratio using least-squares linear regression analysis of peak area ratios versus concentration. Because the slopes of the three calibration curves were not significantly different, the data from the three ratios were combined into a single calibration curve which was used to estimate serum unknowns.

Drug administration

Fasted male volunteers were orally administered one 80-mg verapamil tablet (Isoptin®) simultaneously with 80 mg of d_7-verapamil in 200 ml of water. Blood samples (7 ml) were collected prior to dosing and at specified times post-administration. Serum was isolated by centrifugation and held frozen until assayed.

RESULTS AND DISCUSSION

The degree of separation of a deuterated compound by capillary GC depends on the deuterium content and position [4]. Separation (α) is directly related to the deuterium content. Low deuterium contents in drugs are desirable in order to minimize in vivo isotope effects. d_7-Verapamil has a deuterium content of only 3.03% which corresponds to a molecular weight increase over verapamil of 1.52%. Based upon the known metabolism of verapamil [7] the isopropyl group was assumed to be a metabolically inert region of the molecule. This is necessary if the deuterated analogue is to mimic the in vivo behavior of verapamil. Assuming a linear relationship between α and deuterium content from published data for valproic acid [4], an α-value of 1.014 was estimated for the separation of d_7-verapamil from verapamil. To achieve a resolution of 1.25, $1.3 \cdot 10^5$ effective theoretical plates are required [8]. Several WCOT

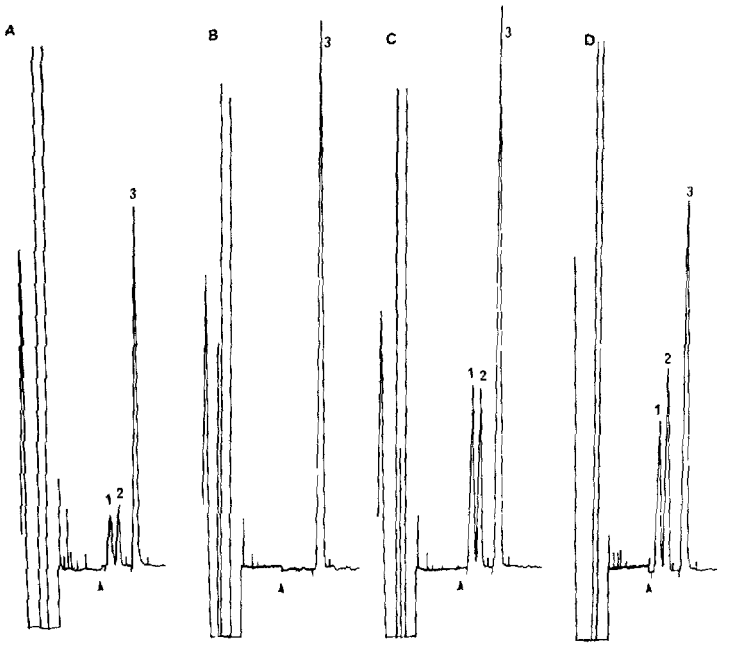

Fig. 1. Chromatograms of serum extracts. (A) Serum standard with verapamil (26.6 ng/ml), d_7-verapamil (27.0 ng/ml) and internal standard; (B) subject serum prior to dosing; (C) subject serum 4 h after dosing; (D) subject serum 1.5 h after dosing. d_7-verapamil (1), verapamil (2) and internal standard (3) have retention times of 20.96, 21.17 and 21.65 min, respectively. Arrow indicates change in attenuation and chart speed from 0.1 to 1 cm/min.

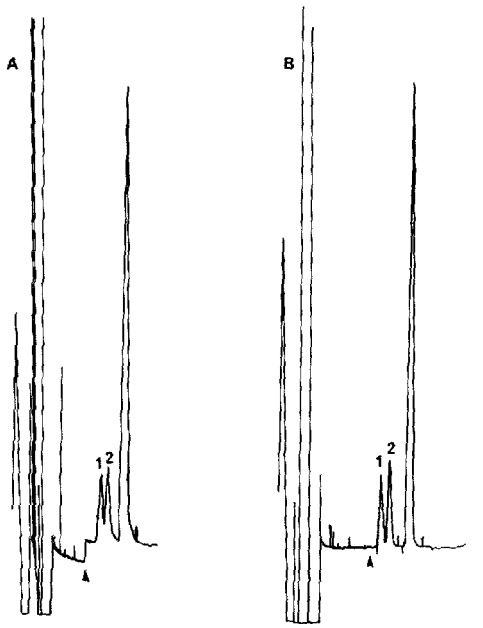

Fig. 2. Chromatograms of a serum standard extract injected before (A) and after (B) cleaning the insert with alkali. d_7-Verapamil (1) and verapamil (2) were present at 27.0 and 26.6 ng/ml, respectively.

columns were tried with resolution less than 1.2. A 60 m × 0.25 mm I.D. thin-film (0.1 μm) bonded WCOT column gave the best results. Peaks were symmetrical with a resolution of 1.3 (Fig. 1). The efficiency was about $1.9 \cdot 10^5$ effective theoretical plates in the splitless mode. Operation in the split mode increased the resolution to 1.4, which corresponds to $4.7 \cdot 10^5$ effective theoretical plates. However, the splitless mode was used to achieve the required sensitivity (5 ng/ml) with automatic sampling. Additional specificity over co-extractives was provided by a nitrogen-specific detector. Vasiliades et al. [9] and Hege [10] have previously combined nitrogen-specific detection with conventional GC to assay verapamil in serum.

It is important to maintain a non-adsorptive injection port insert by using an alkaline cleaning procedure. Fig. 2 illustrates the effectiveness of the alkaline wash procedure in reducing peak tailing.

Reversed-phase extraction columns were found to have a better recovery of verapamil relative to a conventional solvent extraction method. Recoveries of verapamil from serum averaged 81% using an extraction procedure similar to that of Spiegelhalder and Eichelbaum [11] as compared to 96% using a column extraction. One problem with extraction columns is that they occasionally become plugged with fibrin-like material present in serum. This problem was alleviated by dislodging the top filter of the extraction column and allowing liquid to pass around the plugged filter. The column extraction procedure was fast and provided chromatograms free of any interfering peaks (Fig. 1).

Assay precision is given in Table I. Verapamil and d_7-verapamil were at equal concentrations. In the range 16—96 ng/ml, coefficients of variation (C.V.) average 2.9% for both compounds. The minimal detectable concentration is about 1 ng/ml. The minimal quantitative concentration is about 5 ng/ml (C.V. < 25%). To assess accuracy, unknowns were prepared by an independent analyst and assayed. The actual and calculated values are in good agreement as shown in Table II.

The slope parameters of serum standards containing verapamil/d_7-verapamil in ratios of 2:1 and 1:2 did not significantly differ from standards in a 1:1

TABLE I

PRECISION FOR THE ANALYSIS OF VERAPAMIL AND d_7-VERAPAMIL IN SERUM

Actual concentration (ng/ml)	Calculated* concentration (mean ± S.D., n = 3) (ng/ml)		Coefficient of variation (%)	
	Verapamil	d_7-Verapamil	Verapamil	d_7-Verapamil
0	−0.5	0.6		
1.6	1.41 ± 1.06	1.87 ± 0.51	75.2	27.4
4.8	5.47 ± 1.30	5.70 ± 0.97	23.8	17.0
16	15.1 ± 0.82	14.9 ± 1.42	5.40	9.52
32	32.7 ± 0.96	32.0 ± 0.83	2.95	2.60
48	47.9 ± 1.02	47.4 ± 0.97	2.13	2.05
80	80.3 ± 0.25	80.1 ± 1.73	0.31	2.16
96	95.8 ± 0.95	96.4 ± 1.25	1.0	1.25

*By least-squares linear regression analysis (unweighted).

TABLE II

ANALYSIS OF SERUM SAMPLES SPIKED WITH VARIOUS RATIOS OF VERAPAMIL/d_7-VERAPAMIL

Actual concentration (ng/ml)		Calculated concentration (ng/ml)		Absolute error (ng/ml)		Relative error (%)	
Verapamil	d_7-Verapamil	Verapamil	d_7-Verapamil	Verapamil	d_7-Verapamil	Verapamil	d_7-Verapamil
33 8	10.4	35 6	10.8	1.8	0 4	5.2	3.8
13.5	12 5	17 8	13.5	4.3	1 0	27.6	7.7
27.0	75 2	27.3	73 4	0.3	−1 8	1.1	−2 4
54.0	50.2	54.9	52 2	0.9	2 0	1.6	3.9
4.5	12.5	6.1	14 1	1 6	1.6	30.2	12.0
81 0	25.1	85 8	26 2	4 8	1 1	5.8	4.3
18 0	50.2	18 5	50 2	0 5	0	2 7	0
33 8	31.4	32 7	29.4	−1.1	−2.0	−3 4	−6.6
0	0	0 7	0.6	0.7	0.6	—	—
54 0	16.7	53 5	16.4	−0.5	−0 3	−0.9	−1 8
81 0	75.2	79 0	75.2	−2.0	0	−2.5	0
11.2	31.4	12 1	30 2	0.9	−1.2	7.7	−3.9

ratio. Unknowns were calculated for a single calibration curve using the serum standards of all three ratios. The slopes of fifteen calibration curves, determined over a period of two months, averaged 0.00546 ± 0.00023 (4.19%) for verapamil and 0.00542 ± 0.00030 (5.45%) for d_7-verapamil. Correlation coefficients were greater than 0.99.

Human serum samples

Serum samples from a volunteer orally administered 80 mg verapamil · HCl (tablet) and d_7-verapamil · HCl (solution) were assayed under blinded condi-

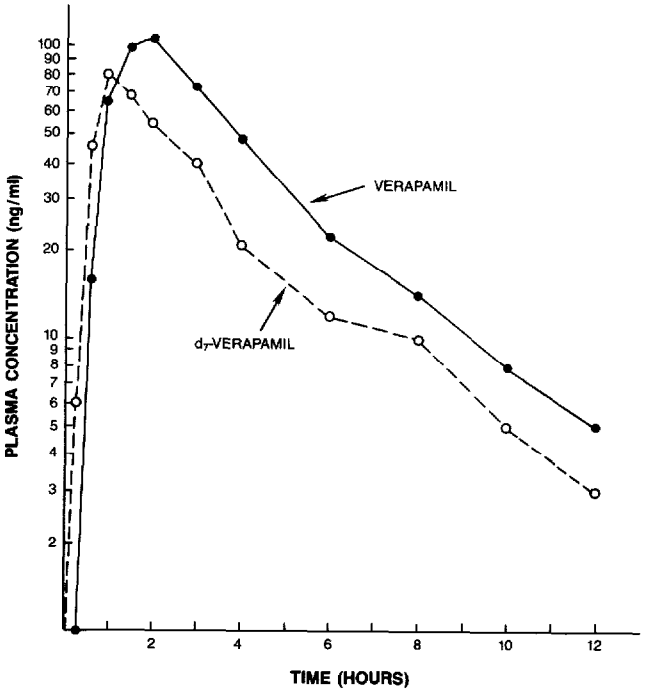

Fig. 3. Verapamil and d_7-verapamil serum concentrations of a subject simultaneously administered a verapamil · HCl tablet (80 mg) and a d_7-verapamil · HCl solution (80 mg in 200 ml water).

tions. The serum concentration—time curve (Fig. 3) indicates that d_7-verapamil concentrations were generally lower than those of verapamil. Generally, if an isotope effect occurs, deuterated drug levels exceed the unlabeled drug levels. The dosage forms assayed within 1% of their specified drug content. This effect is probably related to the extensive first-pass metabolism of verapamil [1]. Additional studies are required to elucidate the cause of this phenomenon.

This novel, stable-isotope analytical technique illustrates that capillary GC is capable of achieving the sensitivity and specificity comparable to GC—MS for deuterated compounds with deuterium contents down to 3%.

ACKNOWLEDGEMENTS

We thank W. Hartmann and R. Stein for the synthesis of d_7-verapamil and Dr. P. Carrigan for his helpful discussions pertaining to the pharmacokinetics of verapamil.

REFERENCES

1 M. Eichelbaum, A. Somogyi, G.E. von Unruh and H.J. Dengler, Eur. J. Clin. Pharmacol., 19 (1981) 13.
2 T.R. Browne, A. VanLangenhove, C.E. Costello, K. Biemann and D.J. Greenblatt, Ther. Drug Monitor., 6 (1983) 3.
3 M. Eichelbaum, H.J. Dengler, A. Somogyi and G.E. von Unruh, Eur. J. Clin. Pharmacol., 19 (1981) 127.
4 D.J. Hoffman and W.R. Porter, J. Chromatogr., 276 (1983) 301.
5 G. Berger, C. Prenant, J. Sastre and D. Comar, Int. J. Appl. Radiol. Isot., 34 (1983) 1525.
6 F. Dengel, U.S. Pat. 3,261,859 (July 19, 1966).
7 M. Eichelbaum, M. Ende, G. Remberg, M. Schomerus and H. Dengler, Drug Metab. Dispos., 7 (1979) 145.
8 W. Jennings, Gas Chromatography with Glass Capillary Columns, Academic Press, New York, 2nd ed., 1980, p. 15.
9 J. Vasiliades, K. Wilkerson, D. Ellul, M. Anticoli and A.P. Rocchini, Clin. Chem., 28 (1982) 638.
10 H.G. Hege, Arzneim.-Forsch., 29 (1979) 1681.
11 B. Spiegelhalder and M. Eichelbaum, Arzneim.-Forsch., 27 (1977) 94.