Journal of Chromatography, 303 (1984) 229–233
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,088

Note

Separation of isotopically labeled vitamin D metabolites by high-performance liquid chromatography

BERNARD P. HALLORAN*, DANIEL D. BIKLE and JOANNE O. WHITNEY
*Departments of Medicine and Pharmaceutical Chemistry, University of California, and *Veterans Administration Medical Center, M/C 11, 4150 Clement Street, San Francisco, CA 94121 (U.S.A.)*

Substitution of either deuterium or tritium for hydrogen in organic compounds can significantly change their physiochemical and/or biological properties[1–6]. For example, substitution of four deuterium atoms for 4 hydrogen atoms in the tetracycline drug Org GC 94 increases its retention time as much as 14% on a μPorasil column[2]. Similar results have also been observed for deuterated benzene and toluene[3] as well as for tritiated prostaglandin E_2 (ref. 4).

Isotopically labeled compounds (both deuterated and tritiated) are extensively used in the study of vitamin D metabolism and the identification of unknown vitamin D metabolites. In most circumstances, it is assumed that the deuterated or tritiated compound behaves physiochemically and biologically in a manner identical to the natural unlabeled compound. However, recent chromatographic evidence from our laboratory[7] and biological evidence from others[8] suggests that the substitution of tritium for hydrogen, in at least some vitamin D compounds, can significantly affect their properties. Recognition of this phenomenon is extremely important in studying vitamin D metabolism. In this paper, we report the separation of two tritium-labeled vitamin D compounds from their unlabeled forms using high-performance liquid chromatography (HPLC).

EXPERIMENTAL

The chemically synthesized, non-radioactive vitamin D metabolites, 1,25-dihydroxyvitamin D_3 (1,25(OH_2D_3), 25(*R*),26-dihydroxyvitamin D_3 (25(*R*),26$(OH)_2D_3$) and 25(*S*),26-dihydroxyvitamin D_3 (25(*S*),26$(OH)_2D_3$) were gifts from M. R. Uskokovic (Hoffmann-La Roche, Nutley, NJ, U.S.A.). Radiolabeled 1,25$(OH)_2$ [23,24(*n*)-^{3}H]D_3 (specific activity 110 Ci/mmole) and 25-hydroxy-[23,24(*n*)-^{3}H] vitamin D_3 (25-OH-[23,24(*n*)-^{3}H]D_3, specific activity 80 Ci/mmole) were purchased from Amersham (Arlington Heights, IL, U.S.A.). Radiolabeled 25,26$(OH)_2D_3$ was biosynthesized from 25-OH-[23,24(*n*)-^{3}H]D_3 using a renal mitochondrial preparation from chicks[9]. This preparation of radiolabeled 25,26$(OH)_2D_3$ has been shown[7] to be predominantly (>90%) 25(*R*),26 $(OH)_2D_3$. All compounds were purified using HPLC before use. Tris-trimethylsilyl derivatives (tris-TMS) of 1,25$(OH)_2D_3$ and 25,26$(OH)_2D_3$ were prepared by reacting the metabolites at 60°C

0021-9673/84/$03.00

for 2 h in pyridine–hexamethyldisilizone with a few drops of trimethylchlorosilane to trigger the reaction[10]. The structural identity of the tris-TMS derivatives of $1,25(OH)_2D_3$, $25(R),26(OH)_2D_3$ and $25(S),26(OH)_2D_3$ were confirmed by mass spectral analysis using direct insertion probe with a Kratos MS-25 mass spectrometer.

HPLC was performed using a Waters HPLC system in conjunction with either μPorasil columns in series (Waters Assoc., Milford, MA, U.S.A.) or two Zorbax-Sil columns in series (DuPont Instruments, Wilmington, DE, U.S.A.). Solvents (HPLC grade) were purchased from J. T. Baker, Phillipsburg, NJ, U.S.A. (hexane, 2-propanol, actonitrile) and Fisher Scientific, Pittsburg, PA, U.S.A. (dichloromethane). They were used either dry (as obtained from the supplier) or after 50% water saturation[11]. Columns were allowed to equilibrate with the appropriate solvent system for 4–8 h before use at a flow-rate of 2–4 ml/min.

To examine the effect of tritium substitution for hydrogen on the chromatographic properties of various vitamin D compounds, a given unlabeled metabolite, or tris-TMS derivative of that metabolite (100–500 ng) was combined with its carbon-23,24(*n*)-tritium labeled counterpart and subjected to HPLC. Retention of unlabeled compound was monitored by UV absorbance at 254 nm while retention of labeled compound was monitored by determining the amount of radioactivity in 15-sec fractions collected across the UV-absorbing peak. Careful attention was paid to correct accurately for the dead volume (1.0 ml) or lag time between the UV monitor and the fraction collector.

RESULTS

Substitution of tritium for hydrogen at carbons-23 and -24 in the tris-TMS derivatives of $1,25(OH)_2D_3$ and $25,26(OH)_2D_3$ significantly increased retention time on HPLC (Figs. 1 and 2, Table I). Utilizing two Zorbax-Sil columns in series, a solvent system of dichloromethane–hexane (15:85), a flow-rate of 2.0 ml/min and recycling twice, tritium labeled $1,25(OH)_2D_3$-tris-TMS eluted at 58 min, 1.25 min after the unlabeled compound (Fig. 1). The separation factor was 1.024. A similar phenomenon occurred with $25,26(OH)_2D_3$-tris-TMS. Application of equimolar amounts of the epimers $25(R),26(OH)_2D_3$-tris-TMS and $25(S),26(OH)_2D_3$-tris-TMS along with a tracer amount of predominantly (>90%) $25(R),26(OH)_2$ [23,24(*n*)-^{3}H]D_3-tris-TMS to two μPorasil columns in series resulted in partial separations of all three compounds (Fig. 2). The retention times for $25(R),26(OH)_2D_3$-tris-TMS, $25(R),26(OH)_2$ [23,24(*n*)-^{3}H]D_3-tris-TMS and $25(S),26(OH)_2D_3$-tris-TMS were 95 min, 98.4 min and 100.6 min respectively. Underivatized vitamin D compounds exhibited similar behavior. The retention time for $1,25(OH)_2$ [23,24(*n*)-^{3}H]D was found to be consistently greater than $1,25(OH)_2D_3$ (Table I).

The degree of separation of tritium-labeled and unlabeled compounds was not the same on all straight-phase systems. For example, application of $1,25(OH)_2D_3$-tris-TMS and $1,25(OH)_2$ [23,24(*n*)-^{3}H]D_3-tris-TMS to two μPorasil columns in series, utilizing a solvent system of acetonitrile–dichloromethane–hexane (0.035:10:90), a flow-rate of 2.0 ml/min and two recycles resulted in a separation factor of 1.011 as compared to a separation factor of 1.024 achieved on Zorbax-Sil columns (Table I).

Maintenance of consistent chromatographic profiles for the tris-TMS derivatives of $1,25(OH)_2D_3$ and $25,26(OH)_2D_3$ is difficult. Initial attempts to study the

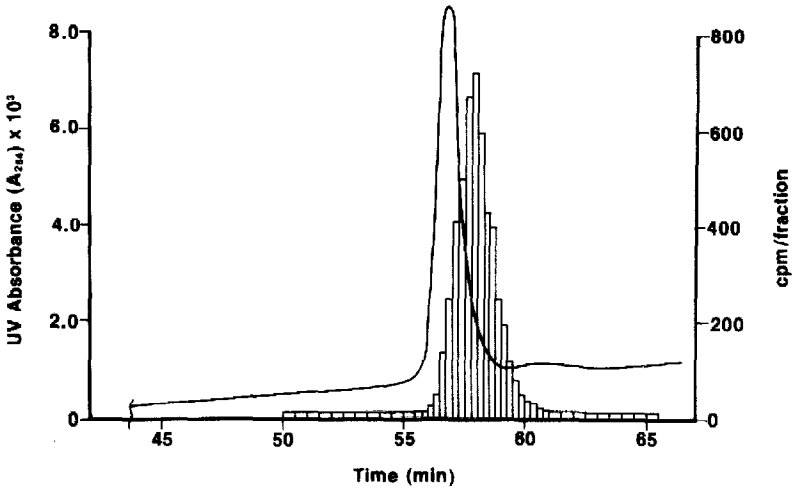

Fig. 1. High-performance liquid chromatogram of 1,25$(OH)_2D_3$-tris-TMS and 1,25$(OH)_2$ [23,24(*n*)-^{3}H]D_3-tris-TMS. Column: two Zorbax-Sil columns in series; solvent: dichloromethane–hexane (15:85); flow-rate: 2.0 ml/min; two recycles. Solid line, UV absorbance; block diagram, cpm.

separation of tritium labeled from unlabeled molecules were frustrated by the inability to attain consistent retention times. One solution to this problem was to use 50%-water-saturated solvents[11]. This technique stabilized retention time but appeared to cause hydrolysis of the TMS ethers, resulting in gradual loss of the compound during chromatography. The most effective means of stabilizing retention time was found to be the addition of a small amount of acetonitrile in the mobile phase. Inclusion of 0.035–0.050% acetonitrile in a solvent system of dichloromethane–hexane (10:90) using μPorasil columns and allowing for adequate equilibration of the column system (4–8 h at 2.0 ml/min) produced consistent results over a 12-month period.

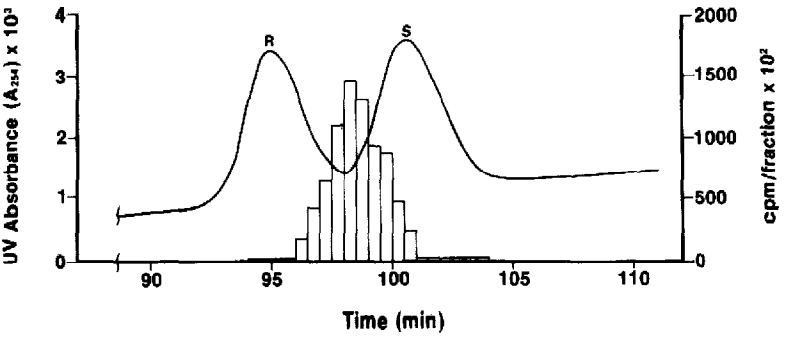

Fig. 2. High-performance liquid chromatogram of 25(*R*),26$(OH)_2D_3$-tris-TMS, 25(*S*),26$(OH)_2D_3$-tris-TMS and 25(*R*)26$(OH)_2$ [23,24(*n*)-^{3}H]D_3-tris-TMS. Column: two μPorasil columns in series; solvent: dichloromethane–hexane (15:85); flow-rate: 4.0 ml/min; two recycles. Solid line, UV absorbance; block diagram, cpm.

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF TRITIUM-LABELED VITAMIN D COMPOUNDS

Chromatography systems: A: two Zorbax-Sil columns; solvent dichloromethane–hexane (15:85); flow-rate 2.0 ml/min; two recycles. B: two μPorasil columns; solvent acetonitrile–dichloromethane–hexane (0.035:10:90); flow-rate 2.0 ml/min; two recycles. C: two μPorasil columns; solvent 2-propanol–hexane (6:94); flow-rate 4.0 ml/min; two recycles. D: two μPorasil columns; solvent dichloromethane–hexane (15-85); flow-rate 4.0 ml/min; two recycles.

Chromatography system	*Sample*	*Retention time (min)*	*Capacity factor, k'**	*Separation factor, α (= k'_1/k'_2)*
A	$1,25(OH)_2D_3$-tris-TMS	56.7	13.19	1.024
	$1,25(OH)_2$ [23,24(*n*)-^{3}H]D_3-tris-TMS	58.0	13.50	
B	$1,25(OH)_2D_3$-tris-TMS	112.8	55.40	1.011
	$1,25(OH)_2$ [23,24(*n*)-^{3}H]D_3-tris-TMS	114.0	56.00	
C	$1,25(OH)_2D_3$	93.4	45.70	1.008
	$1,25(OH)_2$ [23,24(*n*)-^{3}H]D_3	94.2	46.10	
D	25(*R*),26$(OH)_2D_3$-tris-TMS	95.0	46.50	1.037
	25(*R*)26$(OH)_2$ [23,24(*n*)-^{3}H]D_3-tris-TMS	98.4	48.20	
	25(*S*),26$(OH)_2D_3$-tris-TMS	100.6	49.15	

* $k' = (v_x - v_0)/v_0$, where v_0 = void volume of column system and v_x = elution volume of compound.

DISCUSSION

These results indicate that the substitution of tritium for hydrogen in $1,25(OH)_2D_3$, $1,25(OH)_2D_3$-tris-TMS and $25,26(OH)_2D_3$-tris-TMS significantly affects the chromatographic properties of these compounds. In general, introduction of tritium tends to increase retention time using straight-phase chromatographic conditions. This appears to be the case for other compounds as well. Substitution of tritium or deuterium for hydrogen increases the retention time of prostaglandin E_2 and Org GC 94 on straight-phase systems[2,4] and reduces the retention time of benzene, toluene, phenytoin and caffeine on reversed-phase systems[3,5,6].

Recognition of this phenomenon is extremely important when radioactive compounds are used to monitor the elution of non-radioactive compounds from chromatographic systems. One example involves the use of radiolabeled $25,26(OH)_2D_3$ to help identify the naturally occurring stereoisomer of $25,26(OH)_2D_3$. The 25-hydroxyl group of $25,26(OH)_2D_3$ can assume two possible configurations; 25(*R*),$26(OH)_2D_3$ or 25(*S*),$26(OH)_2D_3$. Chromatographic conditions used to separate these stereoisomers can also (as in Fig. 2) separate tritium-labeled from non-labeled $25,26(OH)_2D_3$ and unless appreciated, result in an improper identification of the radiolabeled compound. A second example is the use of radiolabeled compounds to mark the elution position of non-labeled compounds in the preparation of serum samples for assay of the vitamin D metabolites. If the eluted metabolite peaks were collected over too narrow an interval, based on eluted radioactivity, some of the non-radioactive material could be discarded unknowingly, leading to errors in the assay results.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institutes of Health: National Institute of Arthritis, Metabolism and Digestive Diseases, No. AM 30513 and National Institute of Child Health and Human Development, No. HD 13787; and from the Veterans Administration. The authors wish to thank Ms. Marilyn Levens for her expert technical assistance.

REFERENCES

1 M. I. Blake, H. L. Crespi and J. J. Katz, *J. Pharm. Sci.*, 64 (1975) 367.
2 J. J. de Ridder and H. J. M. van Hal, *J. Chromatogr.*, 121 (1976) 96.
3 G. P. Cartoni and I. Ferretti, *J. Chromatogr.*, 122 (1976) 287.
4 J. A. Oats, H. W. Seyberth, J. C. Frolich, B. J. Sweetman and J. T. Watson, in T. A. Baillie (Editor), *Stable Isotopes*, University Park Press, Baltimore, MD, 1978, Ch. 25, p. 281.
5 M. G. Horning, J.-P. Thenot, O. Bouwsma, J. Nowlin and K. Lertratanangkoon, *Advan. Pharm. Ther.*, 7 (1979) 245.
6 J.-P. Thenot, T. I. Ruo, G. P. Stec and A. J. Atkinson, Jr., in A. Frigerio and M. McCamish (Editors), *Recent Developments in Mass Spectrometry in Biochemistry and Medicine*, 6, Elsevier, Amsterdam, 1980, p. 373.
7 D. D. Bikle, B. P. Halloran, J. O. Whitney and B. Hollis, *Biochemistry*, in press.
8 Y. Tanaka and H. F. DeLuca, *Arch. Biochem. Biophys.*, 213 (1982) 517.
9 D. D. Bikle, *Biochim. Biophys. Acta*, 615 (1980) 208.
10 J. Whitney, C. H. L. Shackleton, C. G. Edmonds, A. L. Burlingame and C. J. Piel, in E. R. Klein and P. D. Klein (Editors), *Proceedings of the Third International Conference on Stable Isotopes*, Academic Press, New York, 1979, p. 37.
11 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1979, p. 374.