Journal of Chromatography, 543 (1991) 307–314
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 117

Separation of ^{125}I-labelled derivatives of 5-hydroxy-6,8,11,14-eicosatetraenoic acid

I. MUCHA*, ILDIKÓ PALUSKA-FERENCZ and G. TÓTH
Institute of Isotopes of the Hungarian Academy of Sciences, P.O. Box 77, H-1525 Budapest (Hungary)
(First received July 5th, 1990; revised manuscript received January 14th, 1991)

ABSTRACT

Monoiodinated tyrosine methyl ester, a derivative of 5-hydroxy-6,8,11,14-eicosatetraenoic acid containing ^{125}I in the phenolic *ortho* position was prepared with high specific radioactivity and separated by column chromatography on a Sephadex LH-20 gel. The adsorption behaviour of the labelled product was studied both by adsorption chromatography using a Sephadex LH-20 adsorbent with ethanol–water as a binary eluent and by reversed-phase high-performance liquid chromatography using C_{18}-silica as a stationary phase with aqueous binary eluents containing ethanol, methanol or acetonitrile. In both separation systems, a linear relationship was found between the logarithmic capacity factor or distribution coefficient and the logarithmic concentration of the organic solvent.

INTRODUCTION

Various prostanoids can be labelled with ^{125}I through their histamine and tyramine [1] or tyrosine methyl ester (TME) [1,2] and these derivatives have widespread use in radioimmunoassay (RIA). 5-Hydroxy-6,8,11,14-eicosatetraenoic acid [5-HETE] is a key intermediate produced in the lipoxygenase pathway of arachidonic acid, and therefore the ^{125}I-labelled derivative of this compound may be useful in specific RIA, although no such RIA has previously been reported.

In the course of labelling through TME derivatives radioiodine may be incorporated via electrophilic substitution into the aromatic ring in the 3′- and/or 5′-positions, but only the monosubstituted derivatives are suitable as tracers for RIA. As we have shown previously, adsorption chromatography on Sephadex LH-20 is an efficient method for the isolation, with high specific activity, of the monoiodinated derivatives of prostanoids [3], steroids [4–8] and other bioactive compounds [9–11].

In this paper we report on the adsorption chromatographic separation of ^{125}I-labelled 5-HETE–TME from the inactive parent compound using Sephadex LH-20 as adsorbent, a method that permits a high specific activity suitable for RIA to be achieved.

According to a widely accepted model of adsorption, the displacement of solute molecules from a sorbent involves the participation of a stoichiometric number of solvent molecules used as organic modifier. Solutes that behave according to this stoichiometric displacement model are expected to give linear plots of log (distribution

0021-9673/91/$03.50

coefficient) or log (capacity factor) *vs.* log (concentration of organic solvent), with a slope that reflects the stoichiometric number of solvent molecules participating in displacement.

The adsorption behaviour of various iodinated compounds has been studied previously on Sephadex LH-20 adsorbent and a linear log k *vs.* log X relationship was found with an ethanol–water binary eluent [3–11], where k and X are the distribution coefficient and the molar fraction of ethanol, respectively. On the other hand, numerous investigations related to the mechanism of adsorption in high-performance liquid chromatography have also demonstrated a linear relationship between log k' and log X, where k' is the capacity factor. This relationship, originally verified for normal-phase high-performance liquid chromatography (HPLC) using silica as stationary phase, was extended also to reversed-phase (RP) systems using binary mobile phases consisting of water and an organic solvent modifier [12–16].

In previous work, RP-HPLC was found to be an efficient alternative to Sephadex LH-20 adsorption chromatography for the separation of various ^{125}I-labelled prostanoid derivatives and a similar chromatographic behaviour of these derivatives was observed in RP-HPLC and in Sephadex LH-20 chromatography [17,18].

In this work, the chromatographic behaviour of ^{125}I-labelled 5-HETE–TME was studied using both adsorption chromatography with Sephadex LH-20 as adsorbent and ethanol–water as eluent and RP-HPLC using C_{18}-bonded silica as stationary phase with aqueous binary eluents containing ethanol, methanol or acetonitrile as organic modifier. Highly significant linear relationships for log k *vs.* log X (Sephadex LH-20) and log k' *vs.* log X (RP-HPLC) were obtained. To our knowledge, this paper is the first to report on the linearity of log k' *vs.* log (molar fraction of organic modifier) in RP-HPLC for a representative of an important family of small molecules containing the monoiodo-TME functional group.

EXPERIMENTAL

5-(±)-HETE was synthesized from arachidonic acid according to the method of Corey *et al.* [19] (since the racemic form was used throughout this study, the chirality will not be indicated any further). In order to study the chromatographic behaviour of 5-HETE–TME used for the radioiodination, this compound was synthesized using tritium-labelled TME. [^{3}H]TME prepared as described [20] was diluted with non-radioactive TME to a specific activity of about 100 μCi/mg and coupled to 5-HETE using the carbodiimide method.

5-HETE–TME was labelled with ^{125}I by the use of the chloramine-T method. To 2–3 μg (3–4.5 nmol) of 5-HETE–TME in 50 μl of buffer, 1–2 mCi (0.5–1 nmol) of carrier-free Na^{125}I (Institute of Isotopes, Budapest, Hungary) was added, followed by the addition of 50 μl of 0.5% chloramine-T solution. After 60 s, the reaction was quenched with 100 μl of 0.7% sodium metabisulphite solution. All reagents were dissolved in 50 m*M* phosphate buffer (pH 7.4).

Monoiodinated [^{125}I]5-HETE–TME was isolated from the labelling reaction mixture by column chromatography on a preparative scale. Sephadex LH-20 dextran gel (Pharmacia, Uppsala, Sweden) swollen in distilled water was packed in a column (130 × 10 mm I.D.) to a height of 100 mm. The reaction mixture was administered on

the top of gel and allowed to soak in. After equilibration for 10 min, the elution was started with 150 ml of water, followed by 80–100 ml of 20% ethanol, and the monoiodinated product eluted with 30–35 ml of 30% ethanol. The flow-rate was 45–50 ml/h. In order to suppress the dissociation of the phenolic OH group, which would lead to a decrease in adsorption on dextran gel, the pH was adjusted to 4.0 with 0.1 *M* citrate buffer.

The effluent was passed over an NaI(Tl) scintillation crystal and its radioactivity was counted by a ratemeter and recorded by an *X–Y* plotter.

In order to measure the elution volume of [^{3}H]5-HETE–TME, the effluent was collected with a fraction collector and the ^{3}H radioactivity determined by liquid scintillation counting.

For RP-HPLC separation, a two-pump (LKB, Type 2150) gradient system controlled by an HPLC controller (LKB, Type 2152) was used. An RP-18 (Spheri-5) Aquapore Cartridge column (100 × 4.6 mm I.D.) (Pierce) equipped with a guard cartridge was attached to an NaI(Tl) scintillation crystal. Radioactive samples (0.5–1.0 μCi) dissolved in 5–10 μl of mobile phase were injected through a Rheodyne sample injector equipped with a 200-μl sample loop. The ^{125}I radioactivity of the effluent was counted by a ratemeter attached to the scintillation crystal and registered with a potentiometric recorder (LKB, Type 2210).

The elution volumes in various binary eluents were determined in duplicate. The dead volume (1 ml) was determined using Na^{125}I as the non-retained compound. In preliminary experiments the effect of pH on the retention time was studied in acetonitrile–10 m*M* citrate buffer (60:40, v/v) mobile phase in the pH range 4.0–9.0. As the retention volume remained unchanged throughout this range, the pH was unadjusted for the experiments with different binary eluents of various composition.

RESULTS AND DISCUSSION

Isolation of radioiodinated [^{125}I]5-HETE–TME by Sephadex LH-20 column chromatography

Labelling of 5-HETE–TME with ^{125}I led to the production of a complex reaction mixture. As illustrated in Fig. 1, several labelled products could be detected in the effluent collected from the Sephadex LH-20 column. Omitting the free ^{125}I$^-$ eluting at the dead volume, of the three elution peaks peak 2 was assigned to an unidentified labelled by-product. Peak 3 was attributed on the basis of the immunoreactivity to the monoiodinated [^{125}I]5-HETE–TME. On the basis of the decrease or lack of peak 4, when a large molar excess of target material was employed (*i.e.*, 40–100 nmol of 5-HETE–TME to 1 nmol of Na^{125}I), this peak can be attributed to the disubstituted derivative, [^{125}I]5-HETE–TME.

In order to achieve high specific activity, when a ^{125}I-labelled derivative is intended for use as a tracer in radioimmunoassay, perfect separations from the parent compound is of the utmost importance. When run in the same separation system as that used for the isolation of the labelled compound, tritiated 5-HETE–TME, the parent compound of monoiodinated [^{125}I]5-HETE–TME was found to elute with water near the dead volume. This experiment demonstrated that the monoiodinated product eluted with ethanol would not contain any of the unreacted parent compound. At the same time, however, when water was replaced immediately for the final eluent of

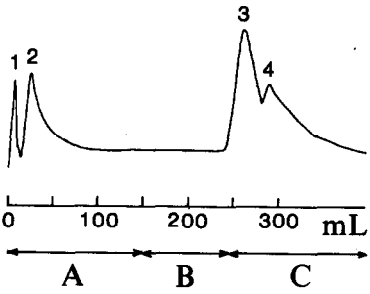

Fig. 1. Separation of ^{125}I-labelled 5-HETE–TME by column chromatography. Sorbent: Sephadex LH-20. Eluent: (A) water; (B) ethanol–water (20:80); (C) ethanol–water (30:70). Peaks: 1 = free $^{125}I^-$; 2 = unidentified labelled product; 3 = [^{125}I]5-HETE–TME; 4 = [$^{125}I_2$]-5-HETE–TME.

30% ethanol, the monoiodinated product to be isolated was usually contaminated with labelled by-products. So as to ensure the removal of these radioactive impurities, the final elution with 30% ethanol was preceded with an intermediate elution using a lower concentration of ethanol. This elution pattern, illustrated in Fig. 1, led to the isolation of monoiodinated [^{125}I]5-HETE–TME free from both the unreacted parent compound and labelled by-products. The efficacy of this separation procedure and the structure of the labelled product were further supported by the specific binding of the separated material to an antibody raised against 5-HETE–bovine serum albumin conjugate.

The monoiodinated [^{125}I]5-HETE–TME thus purified was used for studying its chromatographic properties in both column chromatography on Sephadex LH-20 and RP-HPLC on alkylated silica as the stationary phase.

Chromatographic behaviour on Sephadex LH-20

The chromatographic behaviour of monoiodinated [^{125}I]5-HETE–TME on Sephadex LH-20 was studied using aqueous ethanol eluents. As can be seen from

TABLE I

DEPENDENCE OF ELUTION VOLUME OF ^{125}I-LABELLED 5-HETE–TME ON THE COMPOSITION OF ELUENT

Sorbent: Sephadex LH-20. Eluent: aqueous ethanol–water.

Ethanol concentration		Elution volume, ml	Distribution coefficient
% (v/v)	Molar fraction, X		
35	0.144	126.7	83.05
40	0.172	60.2	37.51
45	0.203	35.8	20.79
50	0.238	21.8	11.20
55	0.276	17.3	8.12

Table I, the elution volume decreased with increasing ethanol concentration. The distribution coefficient (k) was calculated according to the equation

$$k = \frac{v_e - v_0}{w} = \frac{v_e - 5.44}{1.46} \quad (1)$$

where v_e, v_0 and w are the elution volume, the dead volume and the weight of the adsorbent, respectively.

In the range 30–55% (v/v) ethanol, the distribution coefficient as a function of ethanol concentration was calculated according to the equation

$$\log k = \log k_0 - n \log X \quad (2)$$

where X is the molar fraction of ethanol, k_0 is the distribution coefficient extrapolated to 100% ethanol and n is a constant.

As illustrated in Fig. 2, a highly significant (correlation coefficient 0.9945) linear relationship was found, with the regression line

$$\log k = -1.167 - 3.610 \log X \quad (3)$$

Chromatographic behaviour in RP-HPLC

In RP-HPLC using C_{18}-bonded silica as the stationary phase, the chromatographic behaviour of the pure monoiodinated [^{125}I]5-HETE–TME was identical with that observed with Sephadex LH-20 separation, *i.e.*, the retention time decreased with increasing proportion of organic solvent (ethanol, methanol or acetonitrile) in the aqueous binary eluent (Table II). In order to obtain a parameter suitable for direct comparison of RP-HPLC data with those determined for Sephadex LH-20, the

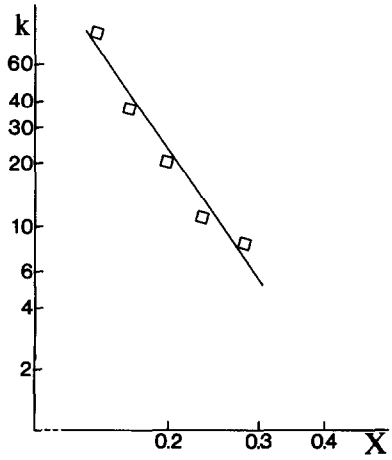

Fig. 2. Distribution coefficient of [^{125}I]5-HETE–TME as a function of ethanol concentration (x) in adsorption column chromatography. Sorbent: Sephadex LH-20. Eluent: ethanol–water. Scale: log–log.

TABLE II

CAPACITY FACTOR OF ^{125}I-LABELLED 5-HETE–TME IN RP-HPLC WITH BINARY ELUENTS

Stationary phase: C_{18}-bonded silica. Mobile phase: aqueous mixtures of organic modifiers.

Concentration				Capacity factor, k'		
% (v/v)	Molar fraction, X			Methanol	Ethanol	Acetonitrile
	Methanol	Ethanol	Acetonitrile			
50		0.238			32.5	
55		0.276	0.296		8.2	17.7
58			0.322			12.7
60		0.319	0.340		5.5	9.7
63			0.369			7.4
65		0.367	0.390		3.0	5.8
68			0.422			4.8
70		0.421	0.445		1.9	3.7
75	0.546		0.508	16.2		2.4
78	0.587			9.4		
80	0.615		0.579	6.7		1.7
82	0.646			4.8		
84	0.677			3.4		
85	0.694		0.661	2.9		1.2

capacity factor, which is directly proportional to the distribution coefficient, was calculated according to the equation

$$k' = \frac{v_e - v_0}{v_0} = \frac{v_e - 1.0}{1.0} \tag{4}$$

where k', v_e and v_0 are the capacity factor, the elution volume and the dead volume, respectively.

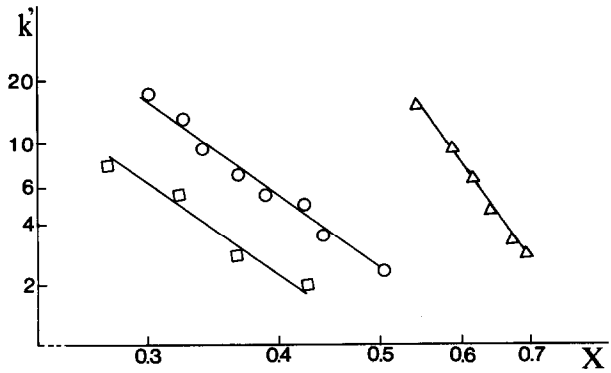

Fig. 3. Capacity factor of [^{125}I]5-HETE–TME as a function of the concentration of organic modifiers (x) in RP-HPLC. Sorbent: C_{18}-bonded silica. Mobile phase: binary mixtures of water and organic solvents (△ = methanol; □ = ethanol; ○ = acetonitrile). Scale: log–log.

Within the approximate capacity factor range 2–10, a linear relationship was found according to the equation

$$\log k' = \log k'_0 - m \log X \quad (5)$$

where X is the molar fraction of organic solvent, k'_0 is the capacity factor extrapolated to 100% organic solvent and m is constant. The regression lines fitted for different binary eluents (Fig. 3) gave the following equations:

$$\log k' = -1.052 - 3.55 \log X \text{ (ethanol)} \quad (6)$$

$$\log k' = -0.715 - 3.68 \log X \text{ (acetonitrile)} \quad (7)$$

$$\log k' = -0.674 - 7.13 \log X \text{ (methanol)} \quad (8)$$

The linearity was highly significant, with correlation coefficients of 0.9966, 0.9980 and 0.9999, respectively.

The widely accepted displacement model of adsorption predicts that solute molecules that behave according to this model will give linear plots of log (concentration) *vs.* log (distribution coefficient) and log (capacity factor). Although it cannot be regarded as direct evidence of a displacement mechanism of solute retention, the linear relationship between the concentration of organic solvents and the capacity factor (eqns. 6–8) observed in our experiments is not inconsistent with this model.

The slope of log k' *vs.* log X curve is said to be an indicator of the strength of a pure solvent used as the mobile phase. The larger slope found with methanol, which leads to a faster decrease in k' with increase in methanol concentration, may underly the good resolution obtained in RP-HPLC of the analogous monoiodo-TME derivatives of various prostanoids using a ternary eluent containing water, acetonitrile and methanol [18]. Further studies could be useful to check how the differences in elution strength could be utilized to optimize the selectivity of the separation of this family of compounds.

Comparing the slopes of fitted lines obtained from the elution on Sephadex LH-20 and RP-HPLC, the ratio was 3.61 (eqn. 3):3.55 (eqn. 6):3.68 (eqn. 7):7.13 (eqn. 8) = 1:1:1:2. The solute showed similar behaviour in terms of the retention dependence on the concentration of ethanol, as indicated by the identical slope of the plots obtained on Sephadex LH-20 (eqn. 3) and C_{18}-bonded silica (eqn. 6). Although the agreement occurred in a different range of composition for the two systems, the results are not inconsistent with the displacement model.

Linearity is normally obtained with capacity factors in the range 0.5–10. Outside this range, at extremes of k', non-linearity is frequently observed and different theoretical approaches were considered to account for this phenomenon [15,16], but none of these interpretations affected the validity of displacement mechanism. The non-linearity was observed in our experiments also in ethanol and acetonitrile below 55 and 60%, respectively, but this phenomenon was not studied in more detail. However, the divergence from linearity observed at extremes of capacity factor is not expected to affect the predictive value of solute retention based on the equations presented, as the linearity was found to be valid in the capacity factor range 2–10, a range that meets the practical requirements for applications of RP-HPLC.

REFERENCES

1 J. Maclouf, M. Pradel, Ph. Pradelles and F. Dray, *Biochim. Biophys. Acta*, 431 (1976) 139.
2 I. Mucha, G. Tóth and B. Tanács, *Izotóptechnika*, 22 (1979) 190.
3 G. Tóth, I. Mucha and B. Tanács, *J. Chromatogr.*, 189 (1980) 433.
4 G. Tóth, M. Wéber and F. Kling, *J. Chromatogr.*, 213 (1981) 511.
5 G. Tóth, *J. Chromatogr.*, 238 (1982) 476.
6 G. Tóth, *J. Chromatogr.*, 267 (1983) 420.
7 G. Tóth and J. Zsadányi, *J. Radioanal. Nucl. Chem. Lett.*, 86 (1984) 25.
8 G. Tóth, *J. Chromatogr.*, 404 (1987) 258.
9 G. Tóth, *Radiochem. Radioanal. Lett.*, 29 (1977) 207.
10 G. Tóth, *Radioanal. Chem.*, 46 (1978) 201.
11 I. Mucha, B. Tanács and G. Tóth, *J. Chromatogr.*, 478 (1989) 280.
12 E. Soczewinski and T. Dzido, *Chromatographia*, 22 (1986) 25.
13 E. Soczewinski, *J. Chromatogr.*, 388 (1987) 91.
14 F. Murakami, *J. Chromatogr.*, 178 (1979) 393.
15 X. Geng and F. E. Regnier, *J. Chromatogr.*, 332 (1985) 147.
16 A. Kalbara, T. Nakagawa, Ch. Hohda, N. Hirata and H. Tanaka, *J. Chromatogr. Sci.*, 27 (1989) 716.
17 I. Mucha and G. Tóth, *J. Chromatogr.*, 438 (1988) 111.
18 I. Mucha and G. Tóth, *J. Chromatogr.*, 483 (1989) 419.
19 E. J. Corey, J. O. Albright, A. E. Barton and S. Hashimoto, *J. Am. Chem. Soc.*, 102 (1980) 1435.
20 I. Mucha, A. Seregi, P. Serfőző, B. Tanács and J. Benkő, *Hung. Pat.*, 193 381 (1987).