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Note

Separation of ^{125}I-labelled prostanoid derivatives by reversed-phase high-performance liquid chromatography

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Prostaglandin molecules labelled with ^{125}I through their histamine and tyramine[1] or tyrosine methyl ester[1,2] derivatives are widely used in radioimmunoassay (RIA). Sephadex LH-20 column chromatography used to separate ^{125}I-labelled prostaglandin E_2 monoiodotyrosine methyl ester from the non-radioactive parent compound[3] enabled the high specific activity required for RIA to be achieved. Recently we have shown that reversed-phase high-performance liquid chromatography (RP-HPLC) can be used as an efficient alternative to Sephadex LH-20 column chromatography for the same purpose[4].

In this study, we extended the RP-HPLC method to the analytical separation of ^{125}I-labelled monoiodotyrosine methyl ester (TME) derivatives of various prostanoids, including thromboxane B_2, from each other and from their non-radioactive parent compounds used as target materials for the preparation of labelled derivatives. We report here the separation of TME and [^{125}I]monoiodo-TME derivatives of 6-ketoprostaglandin F_{1a} (6KPGF), prostaglandin F_{2a} (PGF_{2a}), prostaglandin E_2 (PGE_2), thromboxane B_2 (TXB_2), 13,14-dihydro-15-keto-PGF_{2a} (DHKPGF) and 11-deoxy-13,14-dihydro-15-keto-11B,16E-cyclo-PGE_2 (bicyclo-PGE_2) (Fig. 1).

For this purpose, a water–acetonitrile–methanol ternary eluent was used and the retention values of [^{125}I]monoiodo-TME derivatives as a function of the methanol concentration in the solvent mixture were determined. Isocratic elution proved to be suitable for the separation of the compounds in the polarity range from the 6KPGF to the DHKPGF derivatives. To cover a wider range of polarity, *i.e.*, to include more hydrophobic derivatives in a single run, a linear gradient of methanol was applied. We found that either isocratic or gradient elution RP-HPLC ensured the separation of [^{125}I]monoiodo-TME from unlabelled TME derivatives.

We also studied the effect of low pH on the separation process and found that it decreased the retention time of 6KPGF derivatives (both TME and [^{125}I]monoiodo-TME derivatives), but had only a moderate effect on the retention time of other PG derivatives.

EXPERIMENTAL

Prostanoids were coupled to TME according to general procedure[5] as modified previously for PGE_2 and PGF_{2a}[2]. Bicyclo-PGE_2 was prepared from 13,14-dihydro-15-keto-PGE_2 according to the method of Granström and Kindahl[6].

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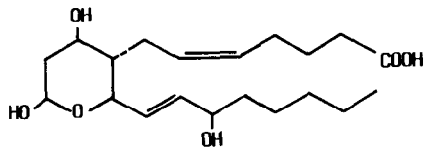

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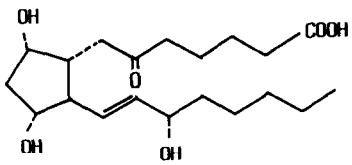

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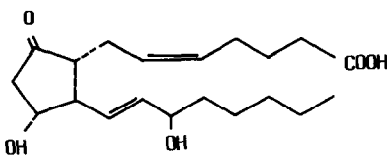
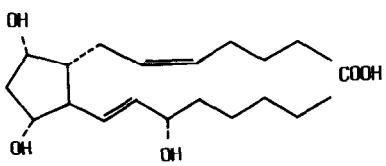
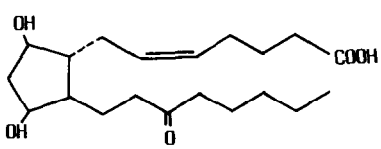


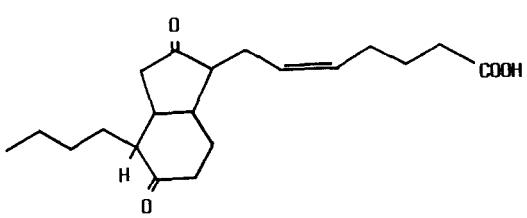
Prostaglandin E_2



Prostaglandin $F_{2\alpha}$



13,14-dihydro-15-keto-$PGF_{2\alpha}$



Bicyclo-Prostaglandin E_2

Fig. 1. Structures of prostanoids.

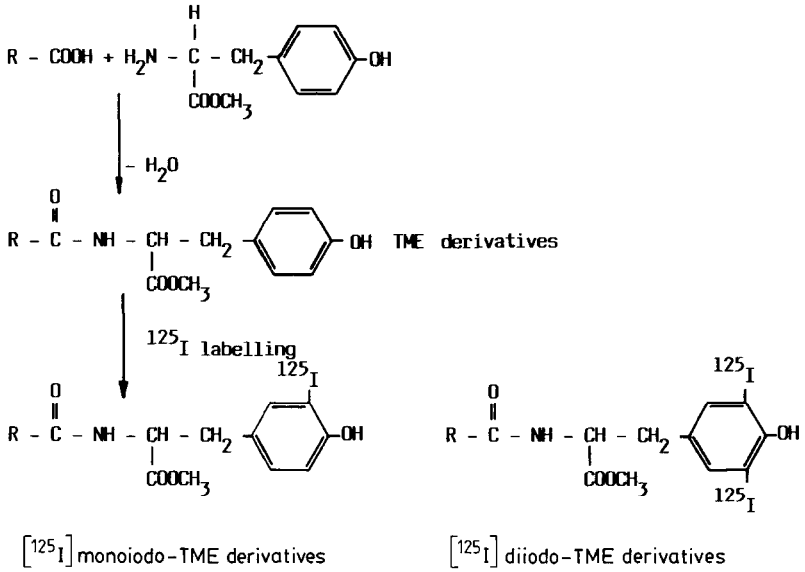


Fig. 2. Reaction scheme for the preparation of [^{125}I]monoiodo-TME derivatives of prostanoids. For RCOOH, see Fig. 1.

TME derivatives were labelled with Na^{125}I by the use of the chloramine-T method and separated for RP-HPLC analysis on Sephadex LH-20 gel by slight modifications of the separation procedure elaborated for TME derivatives of PGE_2 and PGF_{2a}[3].

For RP-HPLC separation, a two-pump (LKB Type 2150) gradient system controlled by an HPLC controller (LKB Type 2152) was used. An Aquapore Cartridge column, RP-18 (Spheri-5), 100 × 4.6 mm I.D. (Pierce) equipped with a guard cartridge was attached on-line to a variable-wavelength monitor (LKB Type 2151) and to a sodium iodide (thallium) scintillation crystal. Non-radioactive samples dissolved in the HPLC solvent and labelled materials dissolved in 30–50% ethanol were injected through a Rheodyne sample injector equipped with a 200-μl sample loop. The solvent system was water–acetonitrile (2:1, v/v) mixed with methanol in various ratios.

UV absorbance was monitored at 280 nm and the gamma radioactivity counted by a ratemeter attached to the scintillation crystal. The two signals were registered with a dual-channel potentiometric recorder (LKB Type 2210).

RESULTS AND DISCUSSION

We determined the elution times of individual ^{125}I-labelled derivatives in the polarity range from the appropriate 6KPGF derivatives to DHKPGF derivatives as a function of methanol concentration in solvent mixtures using isocratic elution. Table I demonstrates that when the solvent contained 45% methanol the separation time was reasonably short (less than 20 min) and the resolution of various derivatives was efficient enough for analytical purposes.

TABLE I

RETENTION VOLUMES (ml) OF VARIOUS PROSTANOID DERIVATIVES

Each derivative was run separately. Solvent A, water–acetonitrile (2:1, v/v); solvent B, methanol. Dead volume, 1.0 ml; flow-rate, 1.0 ml/min.

Prostanoid	*B(%)*					
	40		*45*		*50*	
	TME	*[^{125}I]TME*	*TME*	*[^{125}I]TME*	*TME*	*[^{125}I]TME*
6KPGF	2.5	5.6	1.9	4.5	1.7	3.8
TXB_2	3.8	7.0	2.9	5.4	2.5	4.2
PGE_2	4.6	8.6	3.5	6.4	3.1	4.9
PGF_{2a}	4.8	9.3	3.6	7.0	3.4	5.4
DHKPGF	6.2	12.0	4.7	9.0	4.1	6.3
Bicyclo-PGE_2	9.8	22.9	7.5	16.2	5.2	10.7

The capacity factors calculated from the retention volumes of individual peaks and from the dead volume (1 ml) were at least 1, except for 6KPGF derivatives with eluents containing more than 40% of methanol. The resolution determined by calculating the separation factor according to Meyer[7] was found to be the smallest for PGE_2 and PGF_{2a}, but even in this instance an average resolution of about 1, which led to overlap of neighbouring peaks of only a few per cent[7], could be achieved. This was demonstrated by separation experiments performed with a two-component mixture containing identical amounts of PGE_2 and PGF_{2a} derivatives.

As shown previously[4] for [^{125}I]PGE_2-TME, an RP-HPLC method using isocratic solution with a water–acetonitrile–methanol ternary eluent could be used for the efficient separation of the ^{125}I-labelled monoiodo derivative from the non-radioactive PGE_2-TME. The present observations show that this also holds for all other [^{125}I]monoiodo-TME derivatives and their non-radioactive parent compounds. Moreover, the TME derivatives can be separated not only from ^{125}I-labelled compounds, but also from each other, as demonstrated in Fig. 3 (in order to improve the separation factor for non-radioactive TME compounds, we used 40% methanol in the eluent in these experiments).

It can be seen from Fig. 3 that certain less polar TME derivatives co-migrate with certain more polar [^{125}I]TME derivatives, *e.g.*, PGE_2-TME and PGF_{2a}-TME with [^{125}I]6KPGF-TME and DHKPGF-TME with [^{125}I]TXB_2-TME. This overlap, however, cannot be regarded as a substantial drawback to the utility of this separation system, as in laboratory practice the labelled derivatives are not usually analysed together with the non-radioactive parent compounds. In a normal labelling procedure, the reaction mixture to be analysed contains one unlabelled prostanoid species as a target compound and the labelled derivative(s) produced. In this respect, the separation system is efficient, *i.e.*, there is a baseline separation for each pair of unlabelled–labelled derivatives.

This efficiency allowed us to employ the method for the separation of individual monoiodo derivatives of various prostanoids from their non-radioactive parent compounds also on a peparative scale in a similar manner to that reported previously for PGE_2[4].

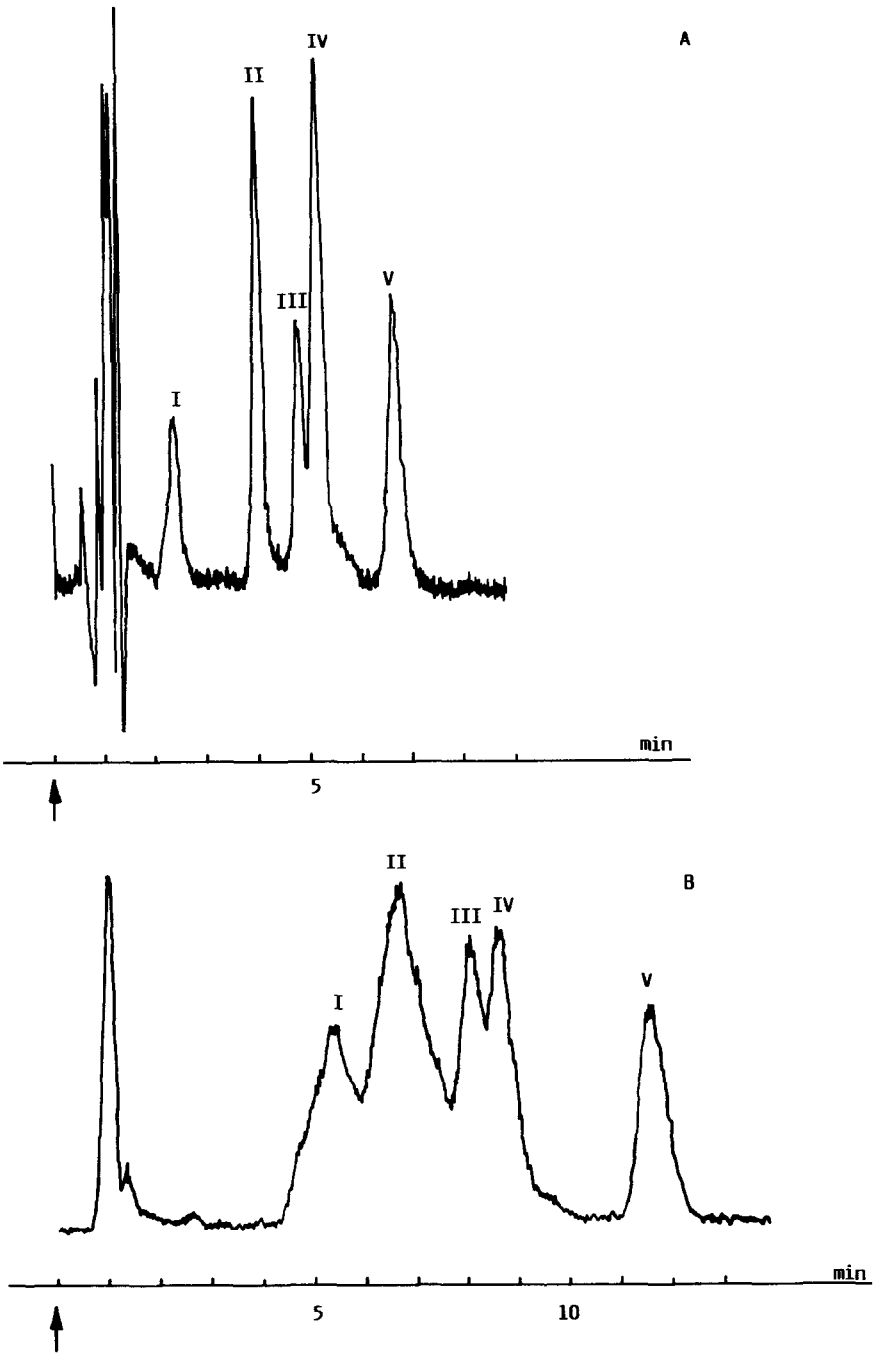


Fig. 3. Separation by RP-HPLC of (A)TME and (B)[^{125}I]monoiodo-TME TME derivatives of prostanoids. [^{125}I]Monoiodo-TME (injected amount 10–20 μl, 0.5–1 μCi) and TME derivatives (injected amount 1–2 μg, 5–10 μl) of 6KPGF (I), TXB_2 (II), PGE_2 (III), PGF_{2a} (IV) and DHKPGF (V) were eluted isocratically with water–acetonitrile (2:1, v/v) containing 40% (v/v) methanol at a flow-rate of 1 ml/min. Free radioiodine elutes with the dead volume at 1.0 min.

Normally during the labelling of a particular TME derivative, one would not expect the conversion of the original prostanoid structure into another type to occur, and therefore the reaction mixture usually cannot contain the [^{125}I]monoiodo-TME derivatives of other types of prostanoids. However, the fact that [^{125}I]monoiodo-TME derivatives of various prostanoids that have only slight structural differences can be separated so efficiently makes it likely that the RP-HPLC method reported here will work even more efficiently in typical real applications, *i.e.*, when the separation from labelled by-products and parent material of only a particular monoiodinated derivative is required.

It can also be seen from Fig. 3 that the band spreads for the radioiodinated compounds are larger than those for the unlabelled compounds. This peak broadening, however, in contrast to that caused by low pH as discussed below, originates from the relatively high volume (50 μl) of the flow cell of the scintillation detector. However, to achieve an appropriate sensitivity at the flow-rate (1 ml/min) involved for as low as 0.5–1 μCi of radioactivity injected, this high volume is necessary. Higher resolution for labelled compounds can be achieved by decreasing the volume of the flow cell and increasing the amount of radioactivity injected.

To decrease the separation time while retaining an acceptable resolution, a linear methanol gradient in the same solvent system can be applied. Gradient elution is also useful with respect to the polarity range of derivatives to be separated. Fig. 4

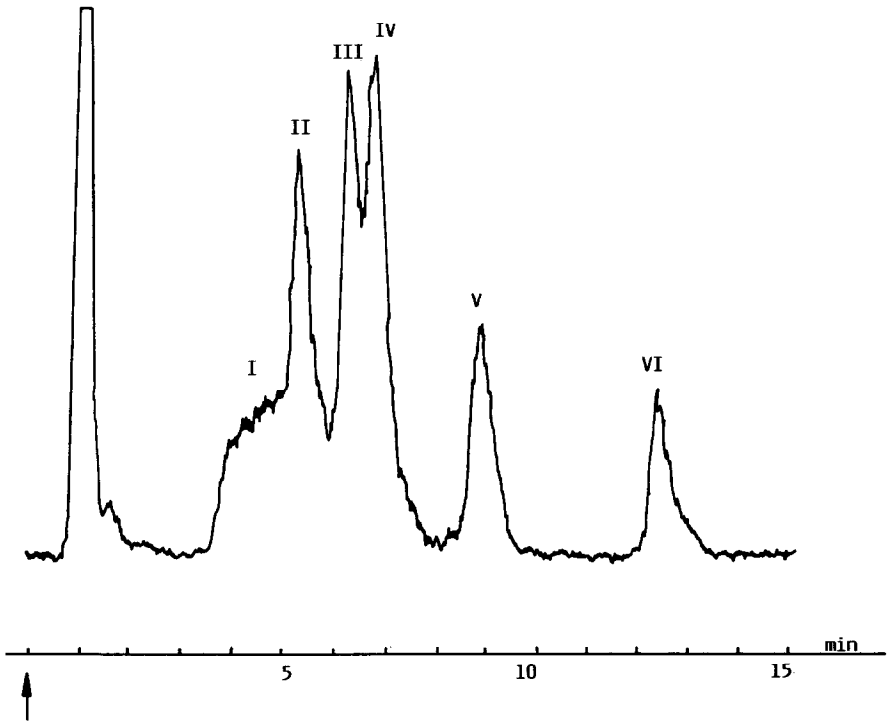


Fig. 4. Separation by RP-HPLC of [^{125}I]monoiodo-TME derivatives using a linear gradient. Elution was started with 45% methanol in water-acetonitrile (2:1, v/v), increasing to 80% from 8 to 13 min. Flow-rate, 1 ml/min. VI = bicyclo-PGE_2; other compounds as in Fig. 3.

demonstrates that the hydrophobic [^{125}I]monoiodo-TME derivative of bicyclo-PGE_2, which would require an inconveniently long separation time in isocratic elution (Table I), can be eluted in less than 15 min in a single run with the more polar [^{125}I]monoiodo-TME derivatives of 6KPGF, TXB_2, PGE_{2a} and DHKPGF. Under these conditions, the elution volumes of the non-radioactive TME derivatives are unchanged (Table I), and therefore the good separation from target compounds of [^{125}I]monoiodo derivatives is subsequently guaranteed. In addition to its use reported here, gradient elution is used routinely in our laboratory for the follow-up of labelling procedures for various protanoid derivatives, so that the retention volumes of the hydrophobic diiodo derivatives produced during the iodination process are decreased.

In contrast to the RP-HPLC separation of free prostanoids, which is generally performed in acidic eluents, with the TME and [^{125}I]monoiodo-TME derivatives of PGE_2 we found it disadvantageous to acidify the ternary eluent[4]. In this work, the effect of pH on the separation time and efficiency was studied further by using the same isocratic solvent system. For this purpose, the solvent was acidified to pH 3.0 with acetic acid. The retention times obtained with 40% and 50% methanol concentrations are summarized in Table II. Comparing these values with those obtained with an unacidified solvent mixture (apparent pH 6.0) (Table I), it can be seen that with a decrease in pH the retention times of both the TME and [^{125}I]monoiodo-TME derivatives of 6KPGF doubled, but only a 10–20% increase was observed with the other prostanoid derivatives. The large increase in retention times of 6KPGF derivatives was accompanied by a simultaneous very strong peak broadening. A pronounced tendency for peak broadening was also observed with other TME and [^{125}I]monoiodo-TME derivatives, although to a much smaller extent than that observed with 6KPGF derivatives. Still, this peak broadening results in an undesirable decrease in the separation factors, and makes it disadvantageous to apply an acidified solvent mixture for analytical purposes.

To explain the significant change in the retention volumes of 6KPGF derivatives, it is worth mentioning that 6KPGF itself is known to isomerize to a hemiketal

TABLE II

RETENTION VOLUMES (ml) IN AN ELUENT MIXTURE OF pH 3.0

Each derivative was run separately. Solvent A, water–acetonitrile (2:1, v/v) containing 0.1% (v/v) of acetic acid; solvent B, methanol. Dead volume, 1.0 ml; flow-rate, 1.0 ml/min.

Prostanoid	*B (%)*			
	40		*50*	
	TME	*[^{125}I]TME*	*TME*	*[^{125}I]TME*
6KPGF	4.3	7.9	3.1	5.3
TXB_2	4.2	7.9	2.7	4.5
PGE_2	5.1	9.9	2.6	4.7
PGF_{2a}	5.6	10.7	3.2	5.2
DHKPGF	7.3	14.2	4.1	6.3
Bicyclo-PGE_2	9.3	27.5	5.2	12.2

form, which results in a peak broadening on silica gel thin layers[8]. It is also interesting that the [^{125}I]histamine derivative of 6KPGF could be isolated in two interconvertible forms, that had the same immunoactivity[9].

These observations raise the possibility that a changed equilibrium at pH 3 between the ketone and hemiketal forms of 6KPGF may account for the significant increase in the retention volume of 6KPGF-[^{125}I]monoiodo-TME that we observed. This phenomenon suggests that the structure of an eicosanoid may play a determining role in the chromatographic properties of [^{125}I]monoiodo-TME derivatives of prostanoids. If so, the contribution to the elution pattern of the TME and [^{125}I]TME derivatives of the parent prostanoid structure may have a beneficial effect with regard to RP-HPLC selectivity. Considering the relatively high pK value of the phenolic OH group in *ortho*-monoiodo derivatives (pK = 8.0), the decrease in pH from 6.0 to 3.0 cannot be considered to affect the ionization pattern of the tyrosine function, but only that of prostanoid skeleton. It can be concluded that it is the definitive role of the prostanoid structure that underlies the good selectivity, which is superior to those of other separation methods, in this RP-HPLC system. Accordingly, we always obtained a better resolution for various derivatives with the RP-HPLC system than with the use of either Sephadex LH-20 adsorption chromatography or silica gel thin-layer chromatography.

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