Journal of Chromatography, 438 (1988) 111–116
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 222

Note

Separation of ^{125}I-labelled prostaglandin E_2-tyrosine methyl ester by reversed-phase high-performance liquid chromatography

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(Received October 12th, 1987)

Prostaglandin molecules, especially prostaglandin E_2 (PGE_2) labelled with ^{125}I through their histamine, tyramine[1] or tyrosine methyl ester[2] derivatives have a widespread use in radioimmunoassay (RIA). To obtain radioiodine-labelled material suitable for use in RIA, *i.e.*, having only one ^{125}I atom per molecule with as high a specific activity as possible, it is necessary to achieve perfect separation of the labelled material from its parent compound. As shown previously, adsorption column chromatography on Sephadex LH-20 gel is an efficient tool not only for the separation of ^{125}I-prostaglandin E_2-tyrosine methyl ester ([^{125}I]PGE_2-TME) from PGE_2-TME[3], but also for the separation with high specific activity of various labelled steroid derivatives[4–8]. Although this method has a lot of advantages over thin-layer chromatography, which is the usual method of laboratory-scale separation, it suffers from the drawback of needing a relatively large amount of solvent and also the separation time is inconveniently long.

The aim of the present work was to elaborate an alternative procedure for the isolation with high specific activity of [^{125}I]PGE_2-TME from the labelling mixture by the use of reversed-phase high-performance liquid chromatography (RP-HPLC), with the expectation that this new method would eliminate the disadvantages of the Sephadex LH-20 procedure.

For this purpose a simple ternary eluent was applied, which proved to be suitable for both monitoring of the labelling reaction and the isolation of pure [^{125}I]PGE_2-TME of high specific activity on a preparative scale. The baseline separation of the monoiodo derivative from other labelled by-products and from the inactive parent compound (PGE_2-TME) was verified by monitoring the β-radioactivity and UV absorbance simultaneously with the ^{125}I-radioactivity. [^{125}I]PGE_2-TME separated by this RP-HPLC procedure proved to be suitable for use as tracer in RIA, as demonstrated by comparing some RIA parameters obtained with this material with those measured with a tracer separated on Sephadex LH-20 gel.

MATERIALS AND METHODS

PGE_2 was coupled to tritium-labelled tyrosine methyl ester (TME) by the carbodiimide method as described previously[2]. [^{3}H]TME was prepared in our laboratory with high specific activity as described[9], and diluted in inactive TME to specific activity about 100 μCi/mg.

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PGE_2-TME was labelled with $Na^{125}I$ by the use of the chloramine-T method and separated on Sephadex LH-20 gel as described[3]. For RP-HPLC separation a two-pump (LKB, Type 2150) gradient system controlled by an HPLC controller (LKB, Type 2152) was used. A μBondapak C_{18} column (300 mm × 3.9 mm, Waters Assoc.) was attached on-line to a variable wavelength monitor (LKB, Type 2151), to a sodium iodide (thallium) scintillation crystal and to a fraction collector (LKB, Super-Rac, Type 2211), respectively. Samples dissolved in the HPLC solvent were injected through a Rheodyne sample injector equipped with a 200-μl sample loop. The solvent system was water–acetonitrile (2:1, v/v) with 30–50% methanol.

UV absorbance was monitored at 280 nm, the γ-radioactivity counted by a ratemeter attached to the scintillation crystal, while β-radioactivity of eluent fractions was determined with a Packard Tri-Carb liquid scintillation spectrometer. γ-Radio activity and UV absorbance was registered simultaneously with a dual-channel potentiometric recorder (LKB, Type 2210).

Experiments with ^3H-labelled materials were made on the same system but separately from ^{125}I-labelled materials.

Anti-PGE_2 antiplasma was developed in rabbits against PGE_2-thyroglobulin conjugate and radioimmunoassay performed according to conventional RIA methods.

RESULTS AND DISCUSSION

Radioiodination of PGE_2-TME by the use of the chloramine-T method en-

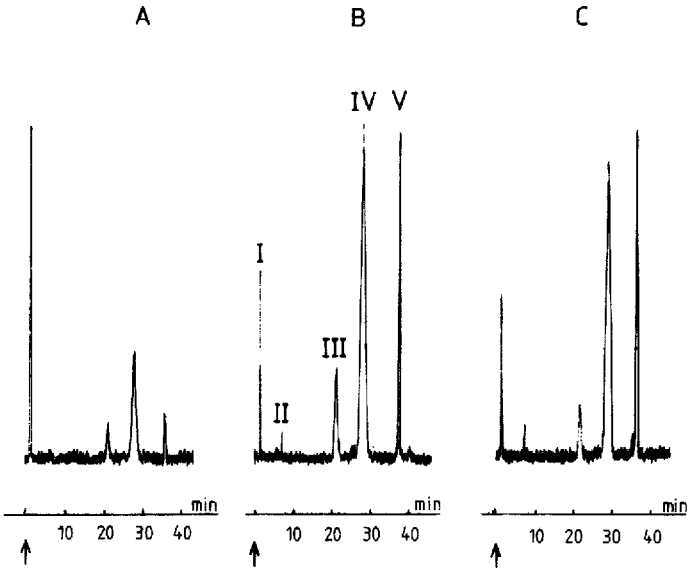

Fig. 1. Time course of radioiodination of PGE_2-TME and the composition of the reaction mixture as monitored by RP-HPLC. A 20-μl volume (0.5 μCi) of reaction mixture after labelling for 5 (A), 60 (B) and 120 s (C) was eluted isocratically with water–acetonitrile (2:1, v/v)–methanol. The methanol concentration was 35% and increased to 60% after 30 min. Flow-rate 1 ml/min. Pressure: 80 bar. Peaks: I = free $^{125}I^-$; II and III = unidentified labelled compounds; IV = monoiodo- and V = diiodo derivative.

sures high incorporation of ^{125}I into the organic molecule. Under our experimental conditions, a labelling time of about 60 s proved optimal. As is seen from Fig. 1, an increase in labelling time failed to improve the yield of monoiodine-labelled target compound.

In full accordance with our previous data, beside free iodine, three different labelled products were observed in the HPLC profile (Fig. 1), which had originated under normal conditions during the labelling procedure. In some instances, however, two further labelled by-products could be detected in large amounts by HPLC, whose retention times differed only slightly from that of the target compound. In these cases the Sephadex LH-20 procedure was inefficient in separating the immunoactive material from these unidentified non-immunoactive by-products, and the tracer thus prepared would give poor results in RIA. However, RP-HPLC separation using isocratic elution with water–acetonitrile–methanol could be applied with good results even in these cases.

For separation on a preparative scale, *i.e.*, from about 100 μCi to a few mCi radioactivity, a reasonably low methanol content, *i.e.*, 30–35%, is suggested in order to achieve a baseline separation of each labelled component even at a relatively high fraction volume *e.g.*, 1 ml.

Data summarized in Table I indicate that the recovery of radioactivity is practically quantitative on a preparative scale.

For routine monitoring of the iodination reaction, an higher methanol content is preferred. Perfect separation of labelled components can be achieved with as high a methanol content as 50% and thus the analysis time can be decreased to about 10–15 min (Fig. 2). This is a valuable benefit as compared, *e.g.*, to the very long separation times for RP-HPLC separation of prostaglandins[10].

During radioiodination the starting material is added to radio-labelled iodine in high molar excess in order that the production of the diiodo derivative is suppressed. Considering this reagent ratio and the separation techniques employed to prepare the monoiodine-labelled derivative for use in RIA, the main point is that the labelled target compound has been separated efficiently from the inactive parent compound. In the case of inefficient separation, a considerable decrease in specific activity is expected which results in a decrease in RIA sensitivity.

However, as illustrated on Fig. 2, with the application of the present RP-HPLC procedure, the difference between the retention time of PGE_2-TME as determined by simultaneous monitoring and measurement of the ^{3}H-radioactivity of

TABLE I

RECOVERY OF RADIOACTIVITY

A labelling reaction mixture (200 μCi) was separated by RP-HPLC with 40% methanol. Flow-rate: 1 ml/min. Fraction size: 1 ml. For peak numbers see Fig. 1.

	Fraction					
	I	*III*	*IV*	*V*	*Remainder*	*Total*
Radioactivity (μCi)	15.8	41.4	77.7	25.6	18.1	178.6
% of total activity	8.8	23.2	43.5	14.3	10.1	100.0

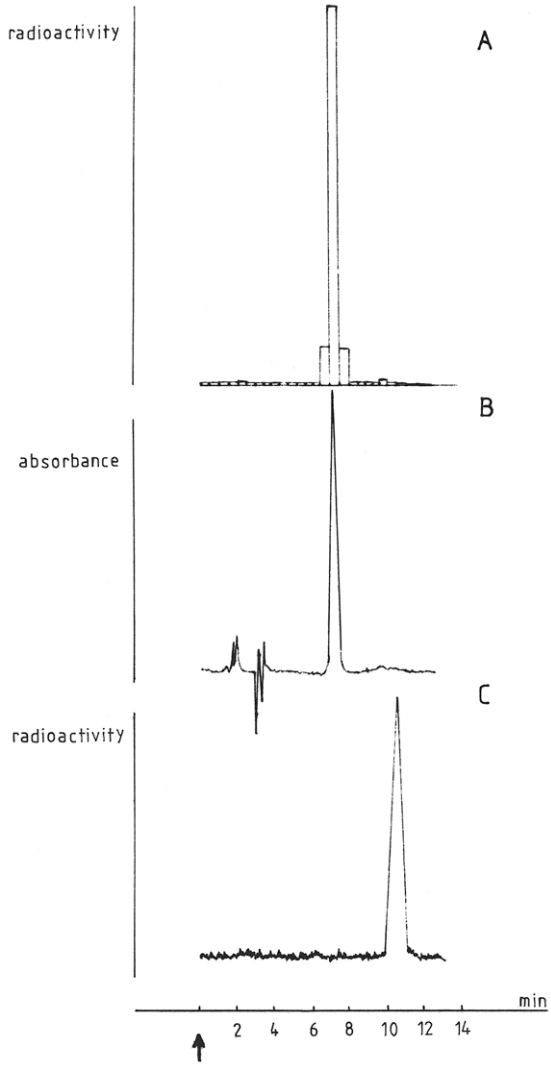

Fig. 2. Separation by RP-HPLC of [^{125}I]PGE_2-TME from PGE_2-TME. Isocratic elution was performed with 50% methanol. (A) ^{3}H-radioactivity of [^{3}H]PGE_2-TME (2 μCi, 20 μl injected); (B) UV absorbance of [^{3}H]PGE_2-TME (2 μg, 20 μl injected); (C) [^{125}I]PGE_2-TME isolated by RP-HPLC from a preparative-scale experiment (0.5 μCi, 10 μl injected). For other parameters see Fig. 1.

[^{3}H]PGE_2-TME and that of [^{125}I]PGE_2-TME confirmed a perfect separation even at an high methanol concentration. Under these conditions, [^{3}H]TME, had a retention time of 6 min, while different prostaglandins were eluted at about 5 min. On a preparative scale, *i.e.*, with a lower methanol concentration, the separation is of course much more effective as a consequence of the increase in retention time of each component.

The adsorption–elution patterns of the individual labelled components are essentially similar in RP-HPLC to that reported previously for Sephadex LH-20 ad-

sorption chromatography[3], *i.e.*, free iodine is eluted with the dead volume, while organic labelled products are eluted in the same sequence as from Sephadex LH-20 gel. This was verified by determining the HPLC retention times of the individual fractions separated on Sephadex LH-20 (not shown). However, contrary to the Sephadex LH-20 technique which differentiates mainly according to the number of iodine atom(s) per molecule[11,12], this RP-HPLC method is also selective according to the structural differences of prostaglandin molecules. It is worth mentioning that data are also available which support an elution pattern of various iodothyronines according to the number of iodine atoms also in RP-HPLC with binary eluents[13]. Contrary to RP-HPLC procedures used for the separation of prostanoids as free acids, with the eluent we applied to the iodinated derivatives the presence of an organic acid in the medium is not required. This lack of acid does not affect the separation efficiency or reproducibility, but eliminates either the pronounced tendency of the radioiodine labelled product to decompose in acidic media or the possibility of conversion PGE_2 into PGA_2.

We also checked the RIA quality of [^{125}I]PGE_2-TME prepared by RP-HPLC separation. As is seen in Fig. 3, the standard dose–response curves obtained with an HPLC tracer and with a tracer separated on Sephadex LH-20 gel, respectively, were essentially identical and there were no significant differences in the typical RIA parameters.

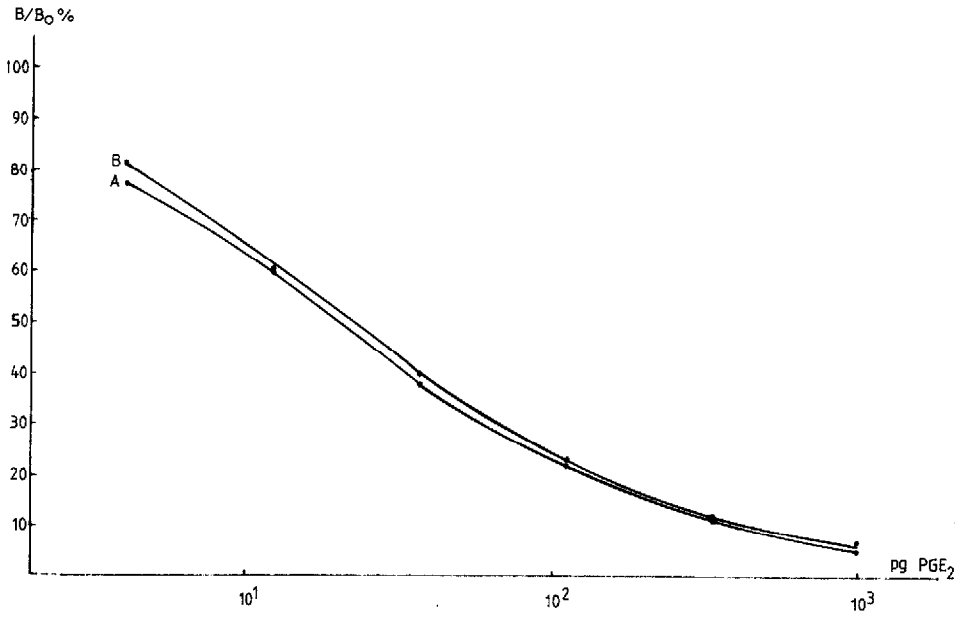

Fig. 3. PGE_2 radioimmunoassay with different [^{125}I]PGE_2-TME tracers. [^{125}I]PGE_2-TME was separated either by RP-HPLC (A) or by Sephadex LH-20 column chromatography (B). Values of the specific binding of tracer in the absence of PGE_2 standard material were 40.4 (A) and 34.7% (B), the non-specific binding expressed as per cent of the total radioactivity was 2.8 (A) and 2.2% (B), while the amounts of PGE_2 necessary to cause 50% inhibition of tracer, *i.e.*, IC-50 values, were 20.1 (A) and 20.8 pg (B) respectively.

While retaining the advantages over thin-layer chromatography of the Sephadex LH-20 column chromatography separation method, *i.e.*, most efficient peak resolution, suitability for use in RIA, effective protection during handling radioactive materials on a preparative scale, etc., this simple RP-HPLC method also makes it possible to save solvent and to decrease the separation time considerably. In addition, it ensures a radioactive concentration high enough for use in RIA even under conventional laboratory conditions, when the total radioactivity used in a single labelling reaction usually does not exceed 100 μCi, a value which is too low to achieve a suitable RIA concentration by the use of column chromatography.

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