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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PROSTACYCLIN

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SUMMARY

A high-performance liquid chromatographic method for the analysis of prostacyclin using a laboratory prepared reversed-phase column packing is described. A relative standard deviation of less than 1% was obtained for ten replicate injections. The system resolves prostacyclin from its hydrolysis product, 6-oxo-prostaglandin $F_{1\alpha}$ and from other prostaglandins present as impurities. These can be estimated to levels of approximately 0.5%. The separation of other unrelated prostaglandins by this method is briefly reported.

INTRODUCTION

Prostacyclin (PGI_2) has been shown[1] to be a potent inhibitor of platelet aggregation. In biological fluids PGI_2 was found to be labile being rapidly converted to 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$)[2]. Fitzpatrick[3] has applied a high-performance liquid chromatographic (HPLC) method to the separation and analysis of prostaglandins as their *p*-nitrophenacyl esters. The preparation of the derivative provided a solution to the problem of the lack of sensitivity for these compounds using detection at a fixed wavelength of 254 nm but with the penalty of a loss of time and precision due to the derivatisation steps in the analysis. Cho and Allen[4] reported that this procedure was not suitable for PGI_2 as it spontaneously decomposed to 6-oxo-$PGF_{1\alpha}$ during resolution on a normal-phase silica gel column as well as on a reversed-phase column. This was presumed to be due to the catalytic power of the acidic adsorption sites on the silica. More recently Fitzpatrick[5] has described a separation of prostaglandins and thromboxanes by gas chromatography using glass capillary columns. This again required a derivatisation step, in this case esterification using diazomethane, in which PGI_2 was determined as the derivative of 6-oxo-$PGF_{1\alpha}$.

HPLC offers advantages of specificity, speed and accuracy for the determination of the acid labile PGI_2 especially if the inherent loss of precision associated with the derivatisation step can be avoided. A simple, precise method has been developed for the analysis of PGI_2 using a variable wavelength detector at about 205 nm, a suitably inert reversed-phase column packing and an inert, UV-transparent mobile phase. The analysis of a typical batch of synthetic PGI_2 sodium salt[6,7] is reported. This

shows the presence of minor impurities whose identities can be postulated by comparison of their retention times with those of the primary hydrolysis product, 6-oxo-$PGF_{1\alpha}$[6,7], and the two Δ^4-isomers[8,9] of prostacyclin. The use of the method for monitoring the stability of PGI_2 is shown. The separation of some other prostaglandins on this system is also reported.

EXPERIMENTAL

Apparatus

A Spectra-Physics Model 740B (Spectra-Physics, Santa Clara, Calif., U.S.A.) motor-driven, reciprocating piston pump employing flow feedback control was used. The detector was a Pye Unicam LC3 (Pye Unicam, Cambridge, Great Britain) variable-wavelength UV detector. Chromatographic columns were operated at ambient temperatures and injections were made using a 50-μl split-flow loop injector as described by Webber and McKerrell[10]. Chromatograms were recorded on a Bryans 28000 (Bryans Southern Instruments, Cheshire, Great Britain) single pen recorder 0–10 mV full scale.

Chromatographic column

The column packing material was prepared by the method given below which is a modification of that supplied by Benezra[11]. 10 μm Partisil (Whatman, Maidstone, Great Britain) (10 g) was dried at 80° *in vacuo* for about 3 h in a 250-ml round-bottomed flask. Octadecyltrichlorosilane (10 ml) and dry toluene (100 ml) were added and the solution refluxed for 3 h with paddle stirring, using a reflux condenser fitted with a calcium chloride guard tube. The mixture was allowed to cool and then filtered through a 0.5-μm Millipore filter (Type FHL PO4700). The silica in the filter was washed with 250 ml methanol slurrying the solid continuously, then with 250 ml hot acetone and dried at 80° *in vacuo* for about 2 h. This gave a carbon loading of about 12%.

The sample (11 g) was refluxed with trimethylchlorosilane (10 ml) and dry toluene (100 ml) for 45 min, washed with methanol and then with acetone and dried *in vacuo* for 2 h to yield the product. This gave an increase in carbon load of about 1%.

A 25 cm by 4 mm I.D. seamless stainless-steel column was packed using carbon tetrachloride as the slurry medium[10].

Reagents

HPLC grade methanol (Rathburn Chemicals, Walkerburn, Great Britain), AnalaR boric acid, AnalaR disodium tetraborate, AnalaR sodium chloride, AnalaR sodium carbonate and reagent grade tetramethylammonium hydroxide (TMAH) (BDH, Poole, Great Britain) were used as received. All prostaglandins were supplied by Dr. N. Whittaker, Wellcome Research Laboratories, Beckenham, Great Britain.

Procedure

Samples for analysis were dissolved in 0.025% (w/v) aqueous TMAH. The stability study was carried out in a carbonate–bicarbonate buffer adapted from that

described by Perrin[12] to be 0.05 *M* in buffer and to have an ionic strength of 0.5 using sodium chloride.

RESULTS AND DISCUSSION

Chromatography

PGI_2 has been reported[4] to undergo specific hydrogen ion catalysed hydrolysis to 6-oxo-$PGF_{1\alpha}$. It was also suggested that the reaction was so facile as to occur on the acidic sites still present on a reversed-phase silica column packing. The method used to prepare the column was therefore designed to give a minimum number of these sites. In order to minimise solute hydrolysis the mobile phase should be as alkaline as possible without stripping off the bonded phase. The mobile phase also had to transmit low-wavelength UV light. Methanol–water mixtures with various buffering systems were tried. Initially ammonium carbonate was employed but the system proved difficult to reproduce whilst a carbonate–bicarbonate buffer system stripped the bonded phase from the column. Finally a boric acid–sodium tetraborate buffer was used and found to be satisfactory.

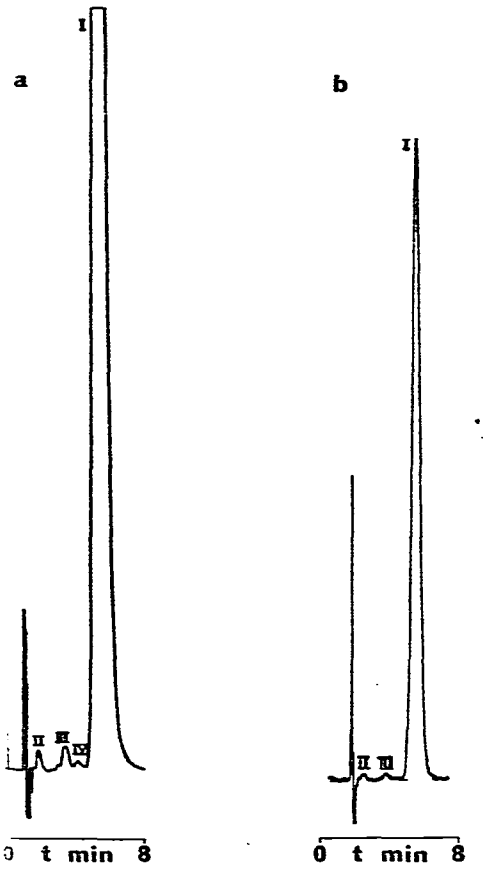

Fig. 1. HPLC of a typical batch of prostacyclin sodium salt. Mobile phase water–methanol (3:2) with 2.5 g l^{-1} boric acid and 3.8 g l^{-1} sodium tetraborate eluent. Flow-rate, 3.6 ml min^{-1}; UV, 205 nm; (a) 0.16 a.u.f.s., 10 μg injected, (b) 0.32 a.u.f.s., 6 μg injected. Structures as in Fig. 2.

The wavelength chosen to monitor the separation, 205 nm, provided the best compromise between maximum sensitivity for PGI_2, which increased as the wavelength was decreased, the efficiency of the detector and interference from the solvent.

Typical chromatograms are shown in Fig. 1. The impurities II, III and IV were found to have the same retention times as the compounds illustrated and named in Fig. 2. The capacity factors (flow-rate 3.6 ml min^{-1}; UV, 205 nm, 0.16 a.u.f.s.; 1 μg injected) of II, III, IV and PGI_2 were 1.4, 2.7, 3.3 and 4.6, respectively; and those at a lower flow-rate (1.5 ml min^{-1}) for PGA_2, PGB_2, PGE_1, PGE_2 and PGI_2 were 4.0, 4.9, 2.7, 2.3 and 4.6, respectively. These data indicate the scope of this approach which requires less sample preparation and only slightly more sophisticated detection equipment than the derivatisation method. Minor modifications to the system should provide analytical methods for use with many prostaglandins.

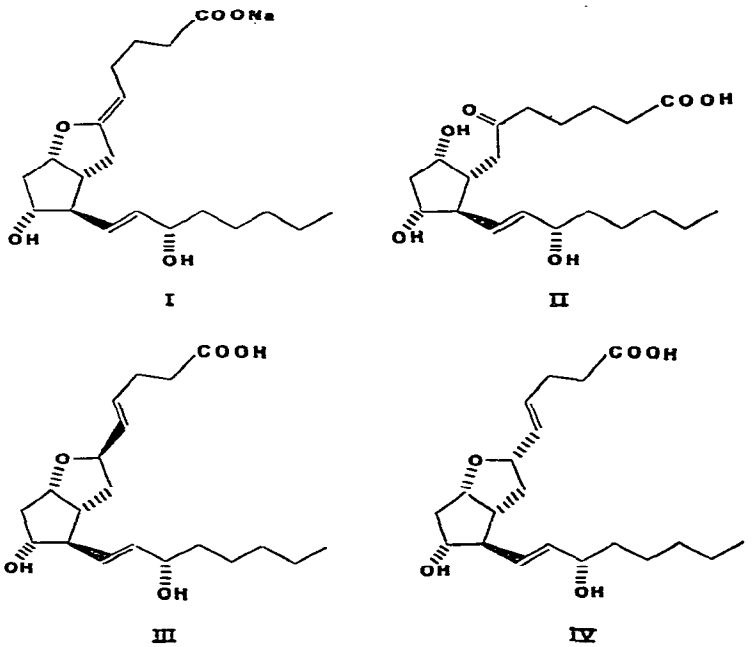

Fig. 2. Structures of prostacyclin and impurities. I = PGI_2 (5*Z*,9α,11α,13*E*,15*S*)-6,9-epoxy-11,15-dihydroxyprosta-5,13-dienoic acid, sodium salt; II = 6-oxo-$PGF_{1\alpha}$ (9α,11α,13*E*,15*S*)-6-oxo-9,11,15-trihydroxyprosta-13-enoic acid; III = (4*E*,6*R*,9α,11α,13*E*,15*S*)-6,9-epoxy-11,15-dihydroxyprosta-4,13-dienoic acid; IV = (4*E*,6*S*,9α,11α,13*E*,15*S*)-6,9-epoxy-11,15-dihydroxyprosta-4,13-dienoic acid.

Calibration

Using peak height measurements the response of PGI_2 was linear in the range 0–50 μg. The relative standard deviation of the peak height for ten successive injections was 0.75% and this method of measurement was found satisfactory for most determinations. The peak height did not decrease significantly over several hours provided the standard was kept at a suitable pH such as that provided by 0.025% TMAH in water.

The small peak height variation indicated that PGI_2 could be analysed by comparing the peak height found in a given sample with that obtained from a

standard solution of an arbitrarily chosen batch of PGI_2 sodium salt and this method has been used to examine the purity and stability of the compound and its formulations.

The purity of a typical batch of PGI_2 was determined by assessing the levels of the observed impurity peaks, using an increased loading and sensitivity to that applied in the analysis of the parent compound as indicated in Fig. 1a. These levels were estimated by peak height against suitable standard solutions as 1% of compounds II and III and 0.5% of compound IV.

Stability

The hydrolytic stability of PGI_2 has been previously examined using a UV spectroscopic method[4]. This HPLC method is ideal for monitoring the decomposition of PGI_2 to 6-oxo-$PGF_{1\alpha}$ and has been carried out, as demonstrated in Fig. 3.

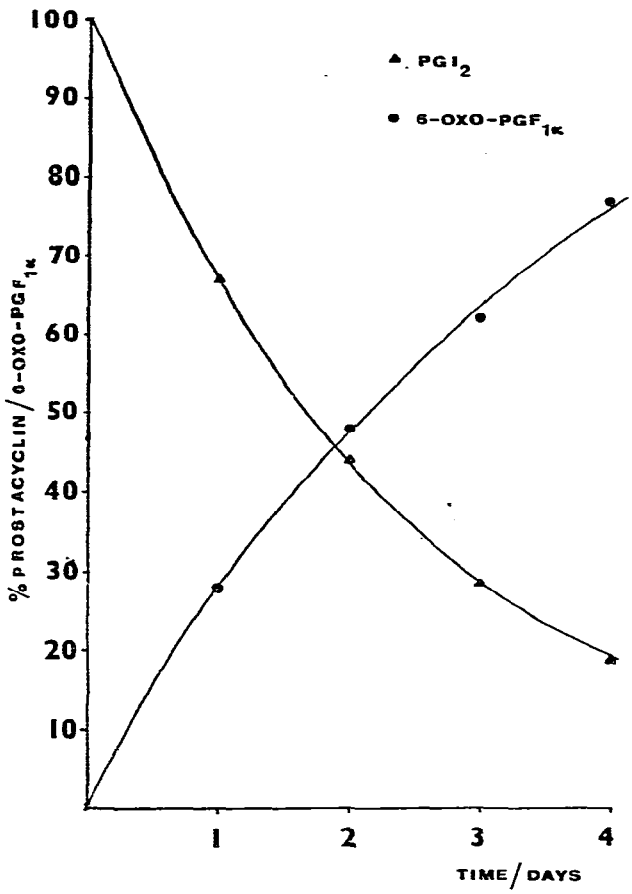

Fig. 3. Stability of prostacyclin at pH 10.5 stored as a 100 μg ml^{-1} solution at 28° in carbonate–bicarbonate buffer. HPLC conditions as in Fig. 1, 2.5 μg injected.

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