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Simultaneous sorption and analytical derivatization on a polystyrene–divinylbenzene polymer Preparation of chromophoric and fluorophoric derivatives of the prostaglandins

Jack M. Rosenfeld*, Xingchun Fang

Department of Pathology, McMaster University, Hamilton, Ontario L8N 3Z5, Canada

Abstract

A solid phase consisting of a macroreticular styrene–divinylbenzene polymer was used as a support for the sorption and derivatization of the prostaglandins to form chromophoric and fluorophoric esters. These highly potent physiological mediators are a group of 20 carbon, oxygenated carboxylic acids which at neutral pH are highly water soluble. In addition to functioning as a support for the simultaneous isolation and derivatization, the solid phase also retained the derivatized analytes while a substantial amount of excess reagent was selectively eluted. The final clean-up was by semi-preparative chromatography.

1. Introduction

Analytical derivatization is an essential requirement for the analysis of compounds such as the prostaglandins (PGs) which possess no inherent electrophore, chromophore or fluorophore. Because of the high sensitivities required, PGs are typically determined by gas chromatography (GC) with mass spectrometric (MS) detection. The GC–MS techniques are well established and have been the basis for numerous investigations in physiology and pharmacology [1–3]. The multi-functional nature of the PGs, however, necessitates a complex sample preparation technique for the determination by GC–MS. Derivatization is a major aspect of these techniques [1–3] and includes: oximation of carbonyls; esterification of the carboxyl group; silylation of hydroxyl groups. All of these steps require anhydrous conditions which can be hard to maintain.

Since the PGs are polar molecules, high-performance liquid chromatography (HPLC) would be a preferred technique. Analysis of PGs by HPLC still requires analytical derivatization but only to provide a group that could be detected by ultraviolet absorption or fluorescence. The additional derivatizations to stabilize the E, D or 6-keto series to increase volatility are not required. Determination of PGs by HPLC as opposed to GC is, therefore, attractive and preparation of chromophoric and fluorophoric

* Corresponding author.

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derivatives of these analytes has been reported [4,5]. These methods, however, are all post-extraction techniques and require both evaporation of the extract to dryness and subsequent reaction in solution. Such sample preparation procedures are relatively difficult to automate. Reactions on solid supports preparatory to instrumental analysis have been studied and described by several groups and are believed to be a very useful approach to automating sample preparations where analytical derivatization is a requirement.

Krull and co-workers [6,7] incorporated reactive groups covalently linked to the surface of a polystyrene–divinylbenzene (PS–DVB) resin. Reactions occur simultaneously with the sorption and only a stoichiometric amount of reagent is used. This technique is used to determine amines which react rapidly with the functional groups on the surface.

Our group at McMaster also utilized a PS–DVB resin but with reagent that had been sorbed onto the surface. In this technique, unmodified, commercially available resin could be used and reagents could be altered at will. For example, to derivatize organic acids preparatory to GC–electron-capture detection (ECD), the analytes were converted to their pentafluorobenzyl (PFB) derivatives by shaking the aqueous sample with XAD-2 that was loaded with PFB bromide (PFBBr) [8,9]. Carbonyls were oximated under the same conditions if the resin was loaded with O-benzylhydroxylamine or O-pentafluorobenzylhydroxylamine [10]. Reactions conditions were completely compatible with aqueous matrix and occurred simultaneously with sorption of the analyte. Evaporation was used to separate excess PFBBr from the non-volatile PFB esters or ethers. Excess oximating reagents were eluted with acid buffer prior to recovery of the oximes.

The technique of using reagents sorbed onto XAD-2 has now been extended to include formation of chromophoric or electrophoric derivatives for HPLC determination of PGs in order to further simplify sample preparation procedures for the study of this important class of compounds.

2. Experimental

2.1. Chemicals

The chromophoric reagent, *p*-bromophenacyl bromide (pBrPhBr) was supplied by Aldrich (St. Louis, MO, USA). The fluorophore, [*p*-(9-anthroyloxy)]phenacyl bromide (PANBr), native PGs and carbon-14 radiolabelled PGs were obtained from Sigma (St. Louis, MO, USA). PFBBr was purchased from Caledon Labs. (Georgetown, Canada). Diisopropylethylamine (DiIPEA) and the solid phase XAD-2 were obtained from BDH (Toronto, Canada) and prepared according to methods previously published.

2.2. Preparation of standards

The ester derivatives which were used as standards were prepared by established procedures [4,5]. The analyte was dissolved in a solution of the esterifying agent (i.e. pBrPhBr, PANBr or PFBBr) and DiIPEA in acetonitrile. This solution was maintained at ambient temperature for 1 h followed by standard work-up [4]. Standards were then further purified by semi-preparative chromatography. The product which was homogenous, as determined by HPLC, was weighed and served as a standard.

2.3. Instrumentation

The pBrPh or the PAN esters were determined on a Waters NovaPak ODS reversed-phase column chromatography with solutions of acetonitrile in water as mobile phases. A Waters M510 pump was used to control flow of the mobile phase and detection was by ultraviolet absorption at 254 nm. The PFB ester of $PGF_{2\alpha}$ was determined on a Hewlett-Packard (HP) 5790 gas chromatograph equipped with J & W telescopic on-column injector, a J & W fused-silica capillary column DB-1, 30 m × 0.321 mm with film thickness of 0.25 μm and a pulse-linearized electron-capture detector.

2.4. Sample preparation

Preparation of a the reaction mixture for derivatization with pBrPhBr

A 1-ml volume of buffer containing PGs or 1 ml biological incubate was added to 4 ml of phosphate buffer at pH 7.4. The resulting 5 ml of analytical sample was added to 300 mg XAD-2 which had been was pre-wetted with 300 μl acetonitrile. A 150-μl volume of a solution containing the esterifying reagent was then added to this heterogenous mixture to commence the reaction.

Preparation of the reaction mixture for derivatization with PANBr.

The reagent was dissolved in acetonitrile at a concentration of 1 mg/ml. A 1-ml volume of this solution was added to 300 mg of XAD-2 followed by 150 μl of trichloroethylene (TCE) and the reaction mixture was shaken for 5 min. A 5-ml volume of water was then slowly added to precipitate the reagent onto the surface of the resin. The liquid phase was discarded. A 5-ml volume of 0.1 *M* phosphate buffer at pH 7.4 and containing the PGs were then added to the resin that had been impregnated with the reagent.

Reaction conditions common to use of both reagents

In one procedure the reaction mixture was shaken at 40°C for 2 h. The resin was isolated by aspiration washed with distilled water. A 10-ml volume of hexane was then added, the mixture was shaken for 10 min and the hexane was removed. The procedure was repeated. The derivatized analyte was eluted with 10% ethylene chloride in diethyl ether which was evaporated to dryness. The residue was taken up in acetonitrile and injected onto the HPLC column. Alternatively the residue was purified by semi-preparative column chromatography (see below).

In the second procedure the shaking was carried out at ambient temperature and the resin was isolated and washed with water as before. Prior to any elution of analyte or excess reagent, the resin was dried under a stream of nitrogen at 40°C. The dried resin was shaken with 10 ml of hexane and the procedure was repeated. Derivatized analytes were eluted and analyzed as described above or were purified by semi-preparative chromatography (see below).

2.5. Determination of yield

By HPLC

A calibration curve was determined by from the responses obtained by injection of known amounts of pure derivative. The recoveries calculated by comparison of the response from injection of an aliquot of the sample.

By recovery of radiolabel

Radiolabelled PGE_2 or $PGF_{2\alpha}$ was added to the reaction mixture and the components of the reaction mixture were separated by semi-preparative chromatography (see below). This procedure had been shown to remove PGs underivatized at the carboxyl group from the esterified products [10]. The yield was then determined from the recovery of radiolabel.

2.6. Semi-preparative chromatography

The residue was taken up in 300 μl dichloromethane and transferred to a 20 × 16 mm Florisil column. The column was washed successively with 20 ml each of hexane, 50% diethyl ether in hexane, diethyl ether and 10% methanol in diethyl ether. Esterified $PGF_{2\alpha}$ was recovered in the last fraction which was concentrated as before. The residue was taken up in a volume of acetonitrile for injection onto the HPLC.

2.7. Measurement of prostaglandins from fibroblast culture

The culture medium contained fibroblasts and 2% fetal calf serum. Three types of samples were run. The first was a blank consisting only of the culture medium. The second sample was a positive control and contained fibroblasts that had been stimulated with interleukin 1 but in the presence of indomethacin to suppress PG production. The third was the experimental sample

Table 1
Effect of mass of XAD-2, structure of reagent and mass of reagent on yield for derivatization of $PGF_{2\alpha}$ from buffer at 40°C for 2 h

	200 mg XAD-2			300 mg XAD-2,
	0.065 mmol (17 mg) PFBBr	0.0036 mmol (1 mg) pBrPhBr	0.0054 mmol (1.5 mg) pBrPhBr	
Yield (%)[a]	90 ± 9	60 ± 13	93 ± 5	96 ± 6

[a] Average ± relative standard deviation ($n = 5$).

which contained fibroblasts stimulated by interleukin 1 to produce PGs.

3. Results and discussion

Solid-phase reaction of PGs with pBrPhBr produced derivatives suitable for HPLC determination but required considerably less reagent than the corresponding reaction with PFBBr (Table 1). Quantitative isolation and pentafluorobenzylation of $PGF_{2\alpha}$ from buffer onto XAD-2 required 0.065 m*M* (17 mg) of PFBBr, a reaction time of 2 h and a temperature of 40°C. Under similar conditions 0.0036 m*M* (1 mg) of pBrPhBr was required to affect similar yield. The fact that less reagent could be used was important because unlike PFBBr, pBrPhBr is involatile and cannot be removed by evaporation. This results in interferences to the determination of these analytes (Fig. 1) and it is evident that a substantial increase in the chromophoric reagent used would produce prohibitive interferences.

One approach to reducing interferences would be to reduce the amount of reagent used in the reaction. This approach was considered because in solution reactions the phenacyl bromides are 1000 times more reactive that the benzyl bromides [11]. With 200 mg of XAD-2, however, reducing the amount of pBrPhBr to 1 mg reduced the yield to 60%. If, however, the mass of XAD-2 was increased to 300 mg then 1 mg of reagent was sufficient to produce quantitative yield. Further reduction in the mass of reagent by increasing the mass of XAD-2 beyond 300 mg

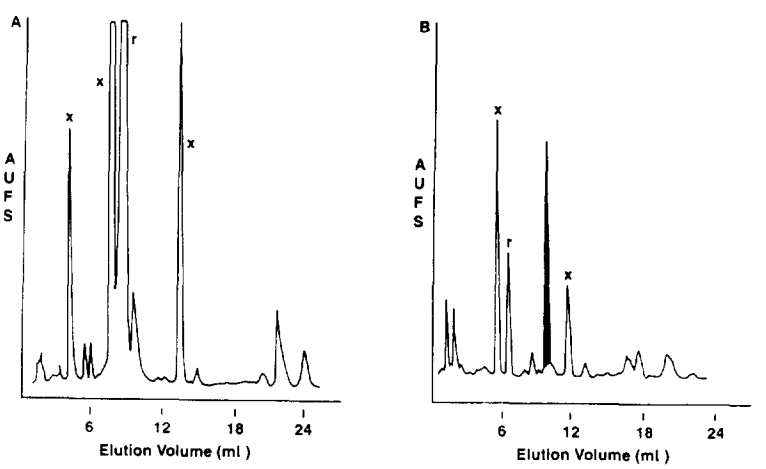

Fig. 1. HPLC traces of the isolates from the derivatization of 5 μg $PGF_{2\alpha}$ with pBrPhBr. (A) Hexane, (B) 10% methanol in diethyl ether. r = Reagent peak; × = peaks from XAD-2.

was not feasible due to difficulty in obtaining efficient mixing in a 100 × 16 mm tube.

These results are consistent with a reaction occurring on the surface of the XAD-2. An increase in surface area would either increase sorption of the analyte or increase the amount of reactive sites. If this is the case then further reduction in the amount of reagent could be obtained by increasing the amount of surface area available in the reaction mixture.

Preparation of the PAN derivative required a modification of the technique for adding the reagent to the reaction mixture. Adding reagent in 150 μl TCE to 300 mg of resin had been previously shown to increase the yield [8] by dispersing the reagent over the total pore volume of the resin bed. PANBr, however, cannot be dissolved at a concentration of 1 mg/150 μl TCE. In consequence, PANBr was co-precipitated with TCE solution onto the resin by the addition of water to a mixture of 300 mg XAD-2 and 1 ml of an acetonitrile solution containing 1 mg reagent and 150 μl TCE. The limited solubility reduced the molar loading of reagent on the surface and as a result the yield of the PAN esters was reduced to 54%.

In order to further reduce the interferences a preliminary clean-up was attempted based on selective elution of the reagent from the XAD-2 with hexane. The reactor bed was treated with hexane immediately after shaking and removed more than 80% of the excess reagent from the XAD-2. In addition, because excess reagent was rapidly removed the reaction was quenched. The derivatized analytes could be subsequently eluted with 10% methanol in diethyl ether (Fig. 1). The removal of excess PANBr was also successfully carried out in this manner (Fig. 2) although there were other interferences in the ultraviolet detection trace possibly arising from the XAD-2.

In an attempt to further improve efficiency for eluting the excess reagent, a variation in the procedure was investigated and this led to an alternative derivatization technique. It was considered that more reagent could be removed by increasing the contact between the XAD-2 surface and the eluting solvent. To this end the

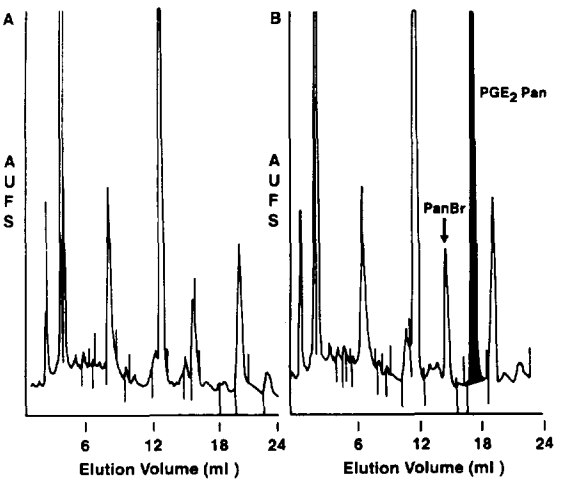

Fig. 2. HPLC traces of the 10% methanol in diethyl ether eluates from XAD-2 for derivatization of 5 μg PGE_2 with PANBr. (A) Blank, (B) sample.

reactor bed was dried in order to remove the water which is a considered necessary for effective contact between lipophilic solvent and the resin.

Under these conditions, it was found that high yield was obtained with reaction times as low as 20 min *and* at room temperature (Table 2). This unexpected finding was attributed to reaction during the drying phase and the sorption of 70% of the native analyte (Table 2). The latter result was surprising. The pH was 7.4 which ensured ionization of the carboxyl group and moreover $PGF_{2\alpha}$ has three hydroxyl groups which would further enhance the water solubility. As a result methods for isolation of PGs that use solid-phase sorption usually require acidification [3]. It appears, however, that there remains sufficient lipophilic character in the PGs to allow substantial sorption onto XAD-2.

Normal-phase semi-preparative column chromatography was used to separate the final amount of excess reagent. After transfer to a Florisil column the interferences were eluted with a solvents or solvent mixtures with low to moderate eluotropic power. The pBrPh ester of $PGF_{2\alpha}$ was eluted with 10% methanol in diethyl ether. This procedure allowed recovery of the derivatives in a clean fraction (Fig. 3).

Table 2
Recoveries of the pBrPhenacyl ester of $PGF_{2\alpha}$ after reaction at ambient temperature using 300 mg of XAD-2

mmol (mg) pBrPhBr	Recovery (%)[a]		
	HPLC		Radiolabel
	Shaking time 20 min	Shaking time 60 min	Shaking time 20 min
0.0036 (1)	79 ± 5	83 ± 6	80 ± 7
0.009 (2.5)	80 ± 6	81 ± 4	80 ± 5
0.036 (10)	100 ± 5	n.d.[b]	n.d.[b]
0	0	0	70 ± 7

[a] Average ± relative standard deviation ($n = 3$).
[b] n.d. = Not done.

Determination of PGs from plasma provides a worst-case scenario of matrix effects. Plasma exerts major effect of yield that can be overcome to some extent by an increase in the amount of reagent used or an increase in reaction time (Table 3). The mechanism of the inhibition is not due to a decrease in sorption since again 70% of the $PGF_{2\alpha}$ is sorbed onto the surface of the resin.

For in vitro studies the simple clean up and reaction at room temperature may prove to be quite sufficient. As shown in Fig. 4 both PGE_2 and $PGF_{2\alpha}$ were formed in the experimental sample where fibroblasts were stimulated with interleukin 1. The two peaks do not appear in the blank or in the control sample where the formation of PG was inhibited by indomethacin.

One major advantage of solid-phase sample preparation is that the technique is more readily amenable to automation. This work demonstrates that a combination of sorption and reaction on solid phase followed by chromatographic purification can be used to prepare PGs for determination by HPLC using either fluorophoric or chromophoric detection. In addition, sorption of PGs is high even at neutral pH and allows reaction without the requirement of agitation. This would further facilitate the application of this technique to automation. Such a development would also facilitate the purification of the

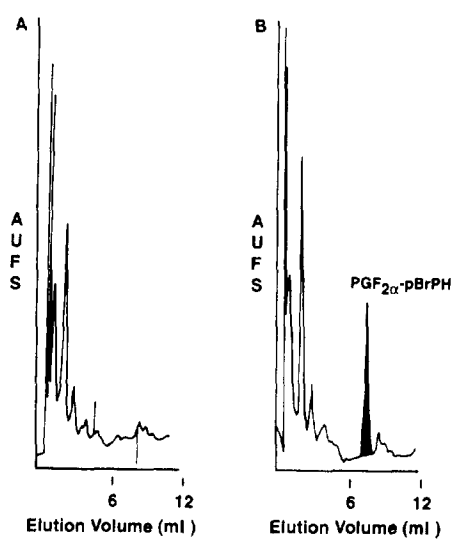

Fig. 3. HPLC traces of the isolate from derivatization of 1 μg $PGF_{2\alpha}$ with pBrPhBr following purification by selective elution and Florisil chromatography. (A) Blank, (B) sample.

Table 3
Recoveries of the pBrPhenacyl ester of $PGF_{2\alpha}$ from plasma as a function of reaction time and reagent

Mass of pBrPhBr (mg)	Recovery (%)[a]	
	Shaking time 20 min	Shaking time 60 min
0.0036 (1)	n.d.[b]	30 ± 8
0.009(2.5)	49 ± 4	50 ± 7

[a] Average ± relative standard deviation ($n = 3$).
[b] n.d. = Not done.

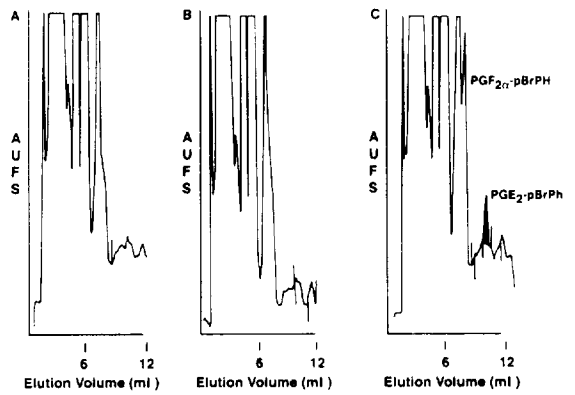

Fig. 4. Extracts of a fibroblast culture. (A) Buffer blank, (B) fibroblasts stimulated with interleukin 1 in the presence of indomethacin, (C) fibroblasts stimulated with interleukin 1.

derivatized analytes which could be done more effectively with automated column switching rather than the manual technique currently in use.

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