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# SOLID-PHASE SAMPLE PREPARATION METHOD FOR PROSTAGLANDINS: INTEGRATION OF PROCEDURES FOR ISOLATION AND DERIVATIZATION FOR GAS CHROMATOGRAPHIC DETERMINATION

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## SUMMARY

A procedure using a solid-phase support has been developed for the isolation and derivatization of prostaglandins from biological matrices. The styrene–divinylbenzene cross-linked copolymeric macroreticular resin, XAD-2, was used as an adsorbent for prostaglandin $E_2$ from biological samples, as a support for the oximation of the carbonyl group and as a catalyst for pentafluorobenzylation. The reactor bed was then linked to a Florisil column for a final chromatographic clean-up. Matrix effects were found to affect the yield, but recovery of the desired electrophoric products was comparable with methods reported in the literature. The ease of sample preparation suggests that this technique may be a viable approach to automating the processes for preparing prostaglandins from biological matrices for gas chromatographic analysis.

## INTRODUCTION

Solid-phase sample preparation has been used to simplify and automate methods in analytical organic chemistry. Current techniques use reversed-phase columns to isolate analytes from aqueous matrices. If necessary, normal phase columns can be used for the further purification of extracts. Isolation and purification, however, are insufficient preparation for those analytes and methods that require derivatization. In such cases reactions on solid supports [1–7] are one approach to developing a complete solid-phase sample preparation scheme. Most such re-

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actions involve a single step, such as formation of an ester, ether or hydrazone. For many analytical problems this is sufficient [8–11] but some compounds, such as certain prostaglandins, require more complex sample preparation. Determination of these analytes by gas chromatography (GC) with electron-capture detection (ECD) or mass spectrometric (MS) detection requires multiple derivatizations [12–21].

Preparation of prostaglandins from biological samples for analysis by GC requires isolation of analyte, oximation, esterification, purification of the reaction products and finally silylation. (The last process, however, simply requires dissolution of final extracts in a silylating solution, which also serves as the solvent to reconstitute the residue for injection onto the column.) In most cases isolation of prostaglandins from the matrix and purification of reaction mixtures can be carried out by solid-phase methods. Subsequently analytes must be isolated and derivatized at the carboxyl and/or the carbonyl group [13–21], which adds to the amount of manipulation necessary for sample preparation. In order to reduce sample handling, derivatization with phase-transfer catalyst [12] was investigated as a means of combining the extraction with one of the derivatization steps. The catalysts, however, were found to interfere with the subsequent instrumental analysis, so they must be removed prior to analysis, which again increases the manipulation required in the procedure.

One approach to resolving these problems would be integration of the solid-supported reactions into the solid sample preparation scheme. We investigated the possibility that XAD-2, a styrene–divinylbenzene cross-linked polymeric macroreticular resin, can be used as a support and/or catalyst for the isolation of analyte from aqueous matrix, oximation and esterification.

## EXPERIMENTAL

### *Chemicals*

Pentafluorobenzyl bromide (PFBBr), methylhydroxylamine hydrochloride (MeHOX) and pentafluorobenzylhydroxylamine hydrochloride (PFBHOX) were purchased from Caledon Labs. (Georgetown, Canada). For derivatization a solution of MeHOX or PFBHOX in pyridine was prepared at a concentration of 10 mg/ml. XAD-2 was purchased from BDH Labs. (Toronto, Canada) and prepared for use as a support by methods described previously [10]. Solvents were obtained from the usual commercial suppliers, such as Fisher and BDH Canada. Prostaglandin $E_2$ ($PGE_2$) and [$^{14}$C]$PGE_2$ with a specific activity of 56.9 mCi/mmol were purchased from Sigma (St. Louis, MO, U.S.A.) and Amersham International (Little Challfont, U.K.) respectively. 6-Ketoprostaglandin $F_{1\alpha}$ (6-$KPGF_{1\alpha}$) was also purchased from Sigma. Florisil was obtained from Supelco (Oakville, Canada).

### *Apparatus*

Derivatized analytes were determined on a Hewlett-Packard 5790 gas chromatograph equipped with a pulse-linearized electron-capture detector, the output of which was monitored and recorded on a Hewlett-Packard HP 3390A integrator. The analyses were carried out on a J&W DB-1 column (30 m$\times$0.23 mm I.D.,

film thickness 0.15 μm). The carrier gas was hydrogen with a linear velocity of 40 cm/min at 200°C. The make-up gas was 10% argon in methane at a flow-rate of 15 ml/min. Samples were injected on-column using a telescopic on-column injector purchased from J&W.

*Derivatization procedure*

Three matrices containing $PGE_2$ were investigated: phosphate buffer, biological incubate (buffer containing 10% fetal calf serum and human lung fibroblasts) and human plasma. A 4-ml volume of aqueous matrix, spiked with $PGE_2$, was added to a 100×16 mm screw-cap vial containing 200 mg of resin. The aqueous phase was acidified with 90 μl of acetic acid. The tube was sealed with PTFE-lined caps, the analyte was adsorbed by shaking at room temperature for 15 min, and the solid phase was isolated by aspiration of the liquid phase using a 20-gauge needle. Then 5 ml of hexane were added to the tube, the mixture was vortexed, the liquid phase was aspirated, and the resin was set in a stream of dry nitrogen and maintained at 60°C for 20 min. Then 200 μl of PFBHOX (or MeHOX) solution were added, the tube was again capped, and the reaction mixture was heated at 60°C for 3 h or overnight at 40°C. The resin was washed with water acidified to pH 3.5 with acetic acid, with the aqueous phase being removed by aspiration. Next, 4 ml of 0.1 *M* phosphate buffer (pH 7.4) were added, followed by 100 μl of a solution of PFBBr in 1,1,2-trichloroethylene (TCE) (1:9, v/v). The tube was capped and shaken for 2 h at 40°C in a water-bath. The resin was isolated by filtration of the reaction mixture through a plug of glass wool in a 5-ml pipetteman purchased from Fisher Scientific (Toronto, Canada).

The mixed derivative of the analytes were isolated by one of two methods. In the first method, designed to recover all the products, the derivatives were eluted from the resin with 10% (v/v) ethanol in diethyl ether [10]. In the second method, used as a purification technique, the resin bed was washed with hexane and then transferred to a 5-ml pipetteman tip containing 1 cm of Florisil, which was topped with 1 cm sodium sulphate. This system, consisting of the resin bed and chromatographic column (referred to hereafter as the link), was washed with 12 ml of methylene chloride and then with 24 ml of 10% ethanol–diethyl ether.

*Thin-layer chromatography*

The ethanol–diethyl ether eluate was evaporated and dissolved in 25 μl of methylene chloride. The entire 25 μl of methylene chloride was transferred to the thin-layer plate on one spot. To ensure a complete transfer of the residue, 25 μl of methylene chloride were again added to the tube and transferred to the plate on the same spot. This procedure was repeated once more. The three transfers assured quantitative spotting of the recovered radiolabel (Table I) and residue. Thin-layer chromatography (TLC) was carried out on silica gel plates, which were developed in the following solvent system toluene–ethyl acetate–methanol (55:45:2.5, v/v). An authentic standard of the mixed derivative was analysed alongside the radiolabelled one. Radiolabelled and authentic derivatives were visualized by autoradiography and iodine staining, respectively [22].

TABLE I

ISOLATION AND DERIVATIZATION OF $PGE_2$ AS THE MIXED PENTAFLUOROBENZYL-OXIME-PENTAFLUOROBENZYL ESTER PRODUCT FROM DIFFERENT MATRICES

| Matrix | Volume of matrix (ml) | $^{14}C$ label added (counts $^{14}C$) | Radiolabel recovered[a] (%) | Total yield[a] (%) |
|---|---|---|---|---|
| Buffer | 4 | 108 204 | 85 ± 8 | 73 ± 4[b] |
| Plasma | 1 | 99 715 | 84 ± 5 | 54 ± 9[b] |
| | | $PGE_2$ (ng/ml) | | |
| Biochemical incubate | 1[a] | 25–250 | | 55 ± 7[c] |
| Plasma | 1[a] | 100 | | 65 ± 7[c] |

[a]Average ± relative standard deviation ($n = 6$).
[b]Yield calculated from total recovery of radiolabel times the fraction of radiolabel that has the same $R_F$ value as the authentic mixed derivative.
[c]Yield calculated relative to extractive alkylation with PFBBr and oximation in solution.

*Gas chromatographic analysis*

The ethanol–diethyl ether eluate from the resin or the link was evaporated to dryness and reconstituted in 50 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)–trimethylchlorosilane (TMCS) (9:1, v/v) and heated for 3 h at 60°C. Prior to injection, the external standard (PFB tetradecanoate) was added to the solution in 10 μl of anhydrous toluene. For 250 ng of $PGE_2$ the isolate was dissolved in 50 μl of BSTFA–TMCS (9:1, v/v), and 0.5 μg of PFB tetradecanoate was added. A 1–2 μl aliquot of this solution was injected using an on-column injection technique. The initial oven temperature was 215°C and this was programmed to 290°C at 4°C/min. There was no time delay at the lower temperature and a 3-min hold at 290°C. The retention time of the minor isomer was 16.73 min, that of the major isomer was 17.53 min and that of the external standard was 12.94 min.

*Calculation of yield*

The yield of the reaction was calculated on the basis of the radiolabel. This was done by first determining the total amount of $^{14}C$ recovered. The radiolabelled material was then separated by TLC of the products. Zones of the plate that contained radiolabel were scraped into counting vials, and the amount of radioactivity was determined by scintillation counting. The fraction of recovered radiolabel that had the same $R_F$ value as the authentic mixed derivative was thus determined. The overall recovery of the product was obtained by multiplying this fraction by the total radiolabel recovered.

For certain experiments the yield was also calculated relative to extractive alkylation from buffer with PFBBr as the reagent and subsequent derivatization with MeHOX or PFBHOX in solution. While not as accurate as the radiolabel method for determining recoveries, this procedure had been previously shown [12] to give a quantitative yield for pure prostaglandin in aqueous solution and was used to conserve the costly $^{14}C$ labelled material.

RESULTS AND DISCUSSION

*Selection of model analyte and derivatives*

The compound selected for extensive investigation was $PGE_2$, which is involved in many physiologically diverse processes [23,24]. The chemical instability and the requirement for high sensitivity makes determination of $PGE_2$ a difficult analytical problem. Moreover, derivatization at two distinct functionalities is obligatory for GC determination. Consequently, this compound was used as the worst-case model analyte.

The sequence of analytical derivatization reactions was oximation of the car-

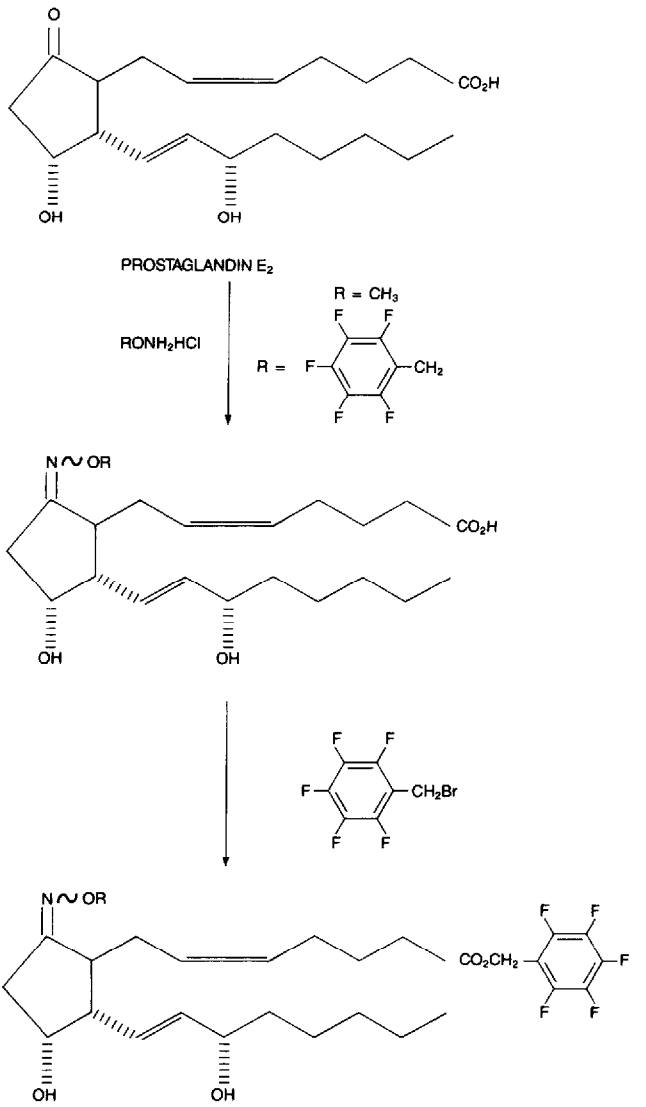


Fig. 1. Derivatization of $PGE_2$ on a solid support.

bonyl group followed by pentafluorobenzylation of the carboxyl group with PFBBr (Fig. 1). Both methoximation and pentafluorobenzyloximation were investigated. If PFBHOX were used for oximation then the reaction product would contain ten fluorine atoms, which would be expected to produce the maximum sensitivity for GC–ECD as well as for GC–MS with negative-ion chemical ionization.

*Reaction yield and effect of matrix*

[$^{14}$C]$PGE_2$ was isolated and derivatized from phosphate buffer as an ideal simple matrix, from biological incubate as a typical matrix and from plasma as a model of a complex biological matrix. Determination of $PGE_2$ from plasma is not a common requirement for physiological or pharmacological investigation, as the physiological concentrations are well below the detection limit of most methods. This matrix was investigated, however, in order to determine the effects that high concentrations of biological components, such as proteins and lipids, might have on the catalytic process or processes.

The total radiolabel recovered from buffer, biological incubate or plasma was ca. 85% (Table I). All of the radiolabel was found in the ethanol–diethyl ether eluate regardless of the initial matrix. Autoradiographic analysis of these isolates showed that, for analyte originating from buffer or plasma, a substantial fraction of the radiolabel corresponded to the mixed derivative. Other compounds detected in minor amounts were starting material and the products of reaction at only the carbonyl group or only the carboxyl group (Fig. 2). Scraping and count-

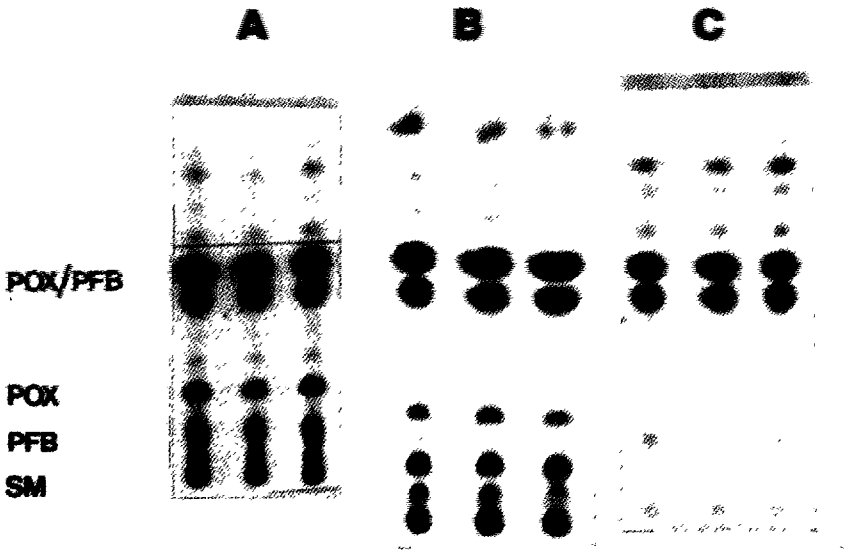


Fig. 2. Typical autoradiographs for preparations of $PGE_2$ originating from (A) buffer, (B) plasma without Florisil clean-up and (C) plasma with Florisil clean-up. Three replicates of each sample matrix are shown. POX/PFB = pentafluorobenzyloxime-pentafluorobenzyl ester derivative with the two spots representing the *syn* and *anti* isomers; POX = pentafluorobenzyloxime derivative; PFB = pentafluorobenzyl ester derivative; SM = starting material.

ing of the radioactive regions of the plate permitted estimation of the amount of material that was recovered as the mixed derivative. For analyte originating from buffer, $73 \pm 4\%$ ($n=6$) of the radiolabel added to the matrix had an $R_F$ value corresponding to the mixed derivative. For $PGE_2$ originating from plasma, $54 \pm 9\%$ ($n=6$) of the material initially added to plasma was found to be the mixed derivative. The remainder of the radiolabel was present in the other regions corresponding to the products of partial reaction.

The reduced recovery from plasma was apparently not due to reduced adsorption of analyte from matrix, since the recovery of radiolabel from all matrrices was 85%. It appears that the reduction is due to a lower reaction yield of both the oximation and the esterification reactions. This was suggested by an increase in the percentage of incomplete reaction products (esterified but not oximated product and oximated but not esterified product) when the procedure was carried out for $PGE_2$ in plasma rather than buffer. This was best demonstrated by scraping and counting the appropriate zones of the TLC plate, which were identified by autoradiography (Fig. 2).

Sulphide has been shown to reduce the yield of XAD-2-supported pentafluorobenzylation of carboxylic acids [10]. Since a major protein of plasma, serum albumin, has sulphydryl groups, it was possible that this may have been the cause of the decreased yield. Consequently $PGE_2$ was isolated and derivatized from an aqueous solution containing physiological concentrations of this protein. The recovery was identical with that obtained for the isolation and derivatization of this analyte from buffer, suggesting that it was not this plasma protein that caused the reduction in yield and that the lower yield may have been due to lipid components or amino acids and peptides that contain sulphydryl groups.

*Effect of in-line chromatographic clean-up*

When clean-up on Florisil was incorporated in the sample preparation procedure the yield of radiolabel from plasma was $67 \pm 9\%$ ($n=6$). In this case, however, scraping and counting of the radioactive regions of the plate (Fig. 2) demonstrated that after clean-up on Florisil a large proportion, $95 \pm 1\%$ ($n=6$) of the recovered radiolabel corresponded to the desired mixed derivative. The overall recovery of the mixed derivative from plasma was thus $63 \pm 5\%$. The material resulting from incomplete reaction or from unreacted starting material was retained on the Florisil column (Fig. 2). More importantly, the clean-up on Florisil also reduced the amount of interference, thus allowing injection of a larger proportion of the final preparation (Fig. 3). Both the *syn* and *anti* isomers (relative to the $\alpha$ chain) were recovered both from the resin and subsequent to purification on Florisil. In both instances the ratio of the *syn* and *anti* isomers (represented by the minor and major peaks, respectively) was similar to that reported in the literature [25].

The yield of the mixed derivative was moderate (Table I) if the matrix was plasma or biochemical incubate. This is common for sample preparation of prostaglandins from biological matrices and when chromatographic purification is involved [21]. It should also be recognized that, in order to isolate the desired derivative, three steps are required: adsorption, oximation and esterification. De-

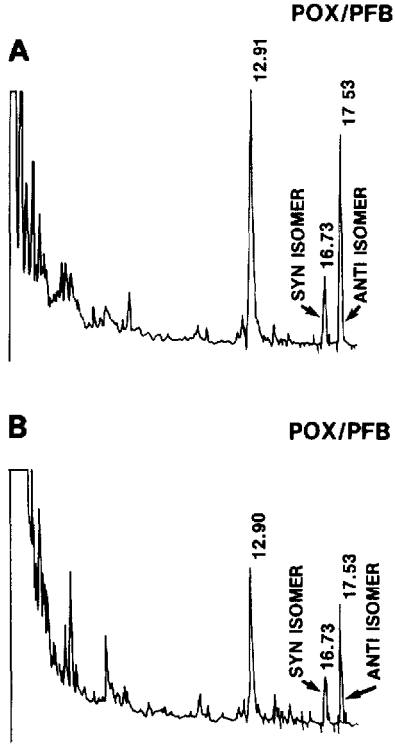


Fig. 3. GC–ECD traces for $PGE_2$ prepared from biological incubate (A) with Florisil clean-up and (B) without Florisil clean-up. The peak at 12.91 min is the calibration standard. Peaks at 16.73 min and 17.53 min are, respectively, the *syn* and *anti* isomers of the mixed POX–PFB derivative.

spite the moderate yield a linear calibration curve was obtained from 25 to 250 ng/ml ($r = 0.996$; slope = 1.02; intercept = 0.01) using the incubation medium as a matrix.

*Derivatization of $6KPGF_{1\alpha}$ and $PGE_2$*

Other reactions of prostaglandins on XAD-2 were also investigated: specifically, that of $PGE_2$ with MeHOX and that of $PGI_2$ with both PFBHOX and MeHOX. The results summarized in Table II indicate that reactions supported on solid XAD-2 can be used to derivatize other carbonyl-containing prostaglandins and to prepare both mixed methoxime and pentafluorobenzyloxime–PFB products of $6KPGF_{1\alpha}$ and mixed methoxime pentafluorobenzyloxime–PFB derivatives of $PGE_2$. This demonstrated that oximation on a solid support is not dependent on the highly fluorinated aromatic ring of PFBHOX or on the location of the carbonyl group on a cyclopentyl ring.

The procedures described have the advantages of all solid-phase sample preparations. Liquid–liquid extraction is not required and thus problems of emulsions and centrifugation do not arise. Since the same adsorbent is used for isolation and two subsequent reactions there is no need for isolation at an intermediate stage of sample preparation. This is required, however, when analytical deriva-

TABLE II

OXIMATION AND PENTAFLUOROBENZYLATION OF $PGE_2$ AND $6KPGF_{1\alpha}$ ON XAD-2

| Analyte | Reaction product | Yield[a] (%) |
|---|---|---|
| $PGE_2$ | MOX-PFB[b] | 65 ± 8 |
| $6\text{-}KPGF_{1\alpha}$ | MOX-PFB[b] | 88 ± 9 |
| | POX-PFB[c] | 74 ± 10 |

[a]Determined relative to extractive alkylation with PFBBr with oximation in solution and stated as average ± relative standard deviation ($n=5$).
[b]Methoxime-pentafluorobenzyl ester derivative.
[c]Pentafluorobenzyloxime-pentafluorobenzyl ester derivative.

tizations are carried out subsequent to solid-phase isolation from an aqueous matrix or in a liquid–liquid procedure. The in-line purification on Florisil further removes the need for additional elutions and evaporations, other than those required in the final purification and isolation. Sample preparation is based on addition, aspiration and filtration of liquids. Such facile procedures are more readily adaptable to automation. This work indicates that reactions supported on XAD-2 can be considered as a rational approach to automating sample preparation techniques, regardless of the complexity of the procedures or the matrix.

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