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# Note

## Separation and determination of prostaglandin $E_1$ metabolites by high-performance liquid chromatography

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Although prostaglandin $E_1$ ($PGE_1$) has been used as a therapeutic agent in arterial occlusive disease for more than a decade, only a few data have been published on the pharmacokinetics and metabolic fate of exogenous $PGE_1$ in man [1–5]. As plasma concentrations of $PGE_1$ in the low pg/ml range and of 15-keto-13,14-dihydro-$PGE_1$ ($KH_2PGE_1$), the major circulating metabolite formed via the 15-hydroxy-PG-dehydrogenase–$\Delta^{13}$-reductase pathway, of the order of less than 1 ng/ml are reached during infusion of the highest tolerable doses of $PGE_1$, highly sensitive and specific determination methods in combination with a reliable chromatographic separation system are essential prerequisites for such studies. In particular, in man the formation of the biologically active 13,14-dihydro-$PGE_1$ ($H_2PGE_1$) during infusion of $PGE_1$ has so far not been described, although the formation of this metabolite *in vitro* [6–8] and in the rat *in vivo* [9] has long been known to occur. It is also of interest that 13,14-dihydro-$PGF_{2\alpha}$ has been found to occur in human plasma after intravenous administration of $PGF_{2\alpha}$ [10,11]. We describe in this paper a specific high-performance liquid chromatographic (HPLC) method for the separation of $PGE_1$ and its primary metabolites and the determination of these PGs in biological material.

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## EXPERIMENTAL

### *Materials*

Acetonitrile (HPLC grade), trifluoroacetic acid (TFA) (HPLC grade), water (HPLC grade), thin-layer chromatographic (TLC) plates and the solvents chloroform, dioxane and acetic acid were purchased from Merck (Darmstadt, F.R.G.). [5,6-$^3$H]$PGE_1$ (60 Ci/mmol) was obtained from New England Nuclear (Dreieich, F.R.G.) and Sep-Pak $C_{18}$ cartridges, $\mu$Bondapak $C_{18}$ [300 mm × 3.9 mm I.D. (10 $\mu$m) and 300 mm × 7.8 mm I.D. (10 $\mu$m)] HPLC columns and Guard-Pak $C_{18}$ precolumns [3.9 mm × 3.9 mm I.D. (10 $\mu$m)] were purchased from Waters Assoc. (Milford, MA, U.S.A.). NAD, NADH and $PGE_1$ were also obtained from Sigma (St. Louis, MO, U.S.A.). Unlabelled standard 15-keto-$PGE_1$ ($KPGE_1$), 13,14-dihydro-$PGE_1$ ($H_2PGE_1$) and 15-keto-13,14-dihydro-$PGE_1$ ($KH_2PGE_1$) were a generous gift from Dr. J. Pike (Upjohn, Kalamazoo, MI, U.S.A.) and were also obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.), respectively. The scintillator fluid Rotiszint 2200 was purchased from Roth (Karlsruhe, F.R.G.).

### *Sample preparation*

Fresh porcine lung obtained from the local slaughterhouse was minced and homogenized in four volumes of 0.1 *M* sodium phosphate buffer (pH 8.0) in a Potter–Elvehjem homogenizer with a loosely fitting PTFE pestle at 1500 rpm for 90 s. A particle-free supernatant was prepared by first centrifuging the homogenate at 4°C at 10 000 *g* for 10 min. The supernatant was then centrifuged at 100 000 *g* for 1 h. After addition of NAD and NADH (final concentrations 1 m*M* each) and [$^3$H]$PGE_1$ (70 000 dpm) to 1 ml of the supernatant, incubations were performed at 37°C for 1 min in a shaking water-bath. The incubations were stopped by acidification with TFA (final concentration 1%) at 0°C and the supernatants were immediately applied to Sep-Pak $C_{18}$ cartridges, which had been activated by washing with 10 ml of acetonitrile followed by 10 ml of 0.1% TFA in water. After washing with 10 ml of 0.1% TFA the cartridges were eluted with acetonitrile–water–TFA (90:10:0.1, v/v). The first eluate fraction of 0.5 ml was discarded, as no radioactivity was eluted. The following 1 ml was collected and the volume was reduced with a stream of nitrogen to 50 $\mu$l and then applied to an analytical HPLC column The $^3$H recovery of the Sep-Pak extraction step was 95.2 ± 1.4% (mean ± S.D., $n=3$).

For preparative purposes, 0.5 mg of $PGE_1$, NAD and NADH (10 m*M* final concentration each) were added to 5 ml of the 100 000 *g* supernatant of porcine lung homogate and incubated at 37°C for 3 h. The incubation was stopped as described above and the supernatant was applied to three Sep-Pak $C_{18}$ cartridges connected in series, which had been activated with 20 ml of acetonitrile followed by 20 ml of 0.1% TFA in water. After application of the sample, the cartridges were washed with 20 ml of 0.1% TFA and eluted as described above. The first

eluate fraction of 1.5 ml was discarded and the following 3 ml were collected, reduced with a stream of nitrogen to 1 ml and then applied to a preparative HPLC column.

*Analytical HPLC*

Reversed-phase HPLC was performed using a Pye Unicam LC-XPD pump connected to a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 injector with a 50-$\mu$l loop using $\mu$Bondapak $C_{18}$ columns and a Guard-Pak $C_{18}$ precolumn. $^3H$ radioactivity in the lung incubates was eluted at 1 ml/min by the following gradient: 0–45 min acetonitrile–water–TFA (30:70:0.05, v/v); 45–70 min acetonitrile–water–TFA (60:40:0.05, v/v). A 7-ml volume of scintillator fluid was added to 1-ml fractions of the eluates and the radioactivity was determined in a Packard Tricarb liquid scintillation spectrometer. Retention times of tritiated $PGE_1$ and its metabolites were compared with those of unlabelled standards. These compounds were monitored by measuring the UV absorption at 222 nm.

*Preparative HPLC*

For the preparative separation of $PGE_1$ metabolites, a $\mu$Bondapak $C_{18}$ column [300 mm × 7.8 mm I.D. (10 $\mu$m)] connected to a Guard-Pak precolumn was used. The gradient and the HPLC equipment with a 1-ml loop was used as described above. PGs were eluted at a flow-rate of 4.0 ml/min. UV absorption at 222 nm was measured. In separate experiments, the retention times of $PGE_1$ and its metabolites were determined using the tritiated compounds as standards.

*Thin-layer chromatography*

[$^3$H]$PGE_1$ and the [$^3$H]$PGE_1$ metabolites generated by incubation and separated by HPLC, together with standards of $PGE_1$, $H_2PGE_1$, $KPGE_1$ and $KH_2PGE_1$, were chromatographed on silica gel 60 TLC plates without fluorescent indicator (20 × 20 cm; layer thickness 0.25 mm). The chromatograms were developed using the solvent system chloroform–dioxane–acetic acid (65:15:3, v/v). The $^3H$ radioactivity on the plates was determined using a Berthold (Wildbad, F.R.G.) LB 2842 automatic TLC linear analyser. The standards were rendered visible by spraying the plates with 4-methoxybenzaldehyde and heating at 110°C for 10 min [12].

*Gas chromatography–mass spectrometry (GC–MS)*

The $PGE_1$ metabolites generated by incubation of 0.5 mg of $PGE_1$ in the 100 000 *g* supernatant of porcine lung homogenate and separated by preparative HPLC were derivatized and subjected to GC–MS. The derivatization procedures and the GC–MS conditions were as described by Schweer *et al.* [13]. In positive-ion chemical ionization (PICI) experiments the ammonia CI gas pressure was 0.9 Torr.

RESULTS

When [$^3$H]PGE$_1$ was incubated in a particle-free porcine lung homogenate in the presence of the cofactors NAD and NADH (1 m*M* each), three main metabolites were rapidly generated. These compounds and the substrate [$^3$H]PGE$_1$ could be clearly separated by the HPLC system used (Fig. 1). The relative amounts of PGE$_1$ and its metabolites found after incubination at 37°C for 1 min were 9.6 ± 3.9% PGE$_1$, 7.0 ± 2.2% H$_2$PGE$_1$, 14.1 ± 7.6% KPGE$_1$, 66.7 ± 8.0% KH$_2$PGE$_1$ and 2.6 ± 1.4% unidentified compounds (means ± S.E M., $n=4$). For validation of the method a TLC solvent system was used, which separates the four PGs sufficiently to demonstrate co-chromatography with the corresponding standard compounds.

As shown in Table I, the three HPLC peaks had $R_F$ values identical with those of standard PGE$_1$ metabolites. Further, when the standards were subjected to HPLC and monitored by UV absorption, the retention times were found to be identical (Table I) with those of the metabolite peaks obtained by incubation of [$^3$H]PGE$_1$ in lung homogenates (Fig. 1).

When the PGE$_1$ metabolites generated by preparative procedures were subjected to GC–MS, KPGE$_1$, KH$_2$PGE$_1$ and H$_2$PGE$_1$ could be formally identified. As an example, the electron-impact pattern of the methyl ester–methoxime–

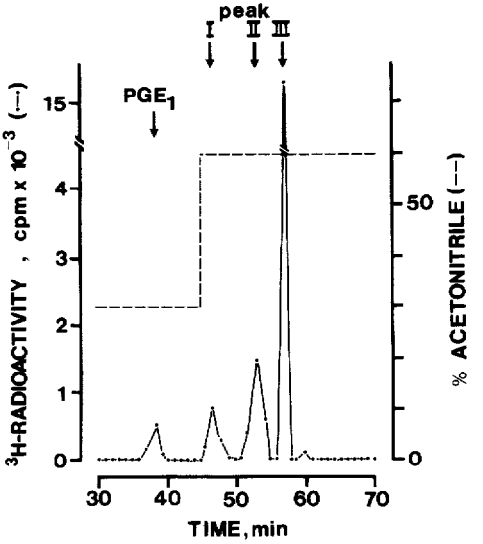


Fig 1. Typical HPLC elution profile of $^3$H radioactivity recovered after incubation of [$^3$H]PGE$_1$ in the 100 000 *g* supernatant of porcine lung homogenate at 37°C for 1 min (NAD and NADH, 1 m*M* each). The arrows indicate the elution positions of 13,14-dihydro-PGE$_1$ (H$_2$PGE$_1$, peak I), 15-keto-PGE$_1$ (KPGE$_1$, peak II) and 15-keto-13,14-dihydro-PGE$_1$ (KH$_2$PGE$_1$, peak III) The dashed line indicates the elution gradient used

TABLE I

$R_F$ VALUES OF $PGE_1$ AND ITS METABOLITES IN TLC AND THEIR HPLC RETENTION TIMES

The $R_F$ values and HPLC retention times of standards are compared with the peaks obtained by HPLC separation of [$^3$H]$PGE_1$ incubated with the 100 000 *g* supernatant of porcine lung homogenate. For the HPLC characteristics of peaks I, II and III, see Fig. 1.

| Compound | $R_F$ value | Retention time (min) |
|---|---|---|
| $PGE_1$ standard | 0.23 | 38 |
| [$^3$H]$PGE_1$ | 0.23 | 38 |
| $H_2PGE_1$ standard | 0.35 | 47 |
| [$^3$H]peak I | 0.35 | 47 |
| $KPGE_1$ standard | 0.54 | 53 |
| [$^3$H]peak II | 0.54 | 53 |
| $KH_2PGE_1$ standard | 0.59 | 57 |
| [$^3$H]peak III | 0.59 | 57 |

trimethylsilyl ether of $H_2PGE_1$ of the second oxime isomer is shown in Table II. The electron-impact mass spectrum is illustrated in Fig. 2. The positive-ion chemical ionization mass spectrum (not shown) contained a signal with *m/z* 544 which represents the molecular ion peak ($[M+H]^+$).

TABLE II

ELECTRON-IMPACT PATTERN OF THE METHYL ESTER–METHOXIME–TRIMETHYLSILYL ETHER DERIVATIVE OF 13,14-DIHYDRO-$PGE_1$ ($H_2PGE_1$)

| Ion assignment[a] | *m/z* | Ion assignment[a] | *m/z* |
|---|---|---|---|
| $[M]^+$ | 543 | $[M-d]^+$ | 342 |
| $[M-CH_3]^+$ | 528 | $[M-OCH_3-2TMSOH]^+$ | 332 |
| $[M-OCH_3]^+$ | 512 | $[M-a-e-CH_3OH]^+$ | 324 |
| $[M-a]^+$ | 472 | $[M-d-OCH_3]$ | 311 |
| $[M-TMSOH]^+$ | 453 | $[M-b-TMSOH]^+$, $[M-c-TMSOH]^+$ | 280 |
| $[M-a-CH_3OH]^+$ | 440 | $[M-a-e-OTMS]^+$ | 267 |
| $[M-OCH_3-TMSOH]^+$ | 422 | $[M-d-TMSOH]^+$ | 252 |
| $[M-b]^+$, $[M-c]^+$ | 370 | $[f]^+$ | 199 |
| $[M-2TMSOH]^+$ | 363 | $[b]^+$, $[c]^+$ | 173 |

[a] a = C-16–C-20; b = C-15–C-20; c = C-9–C-11, d = C-13–C-20; e = C-10–C-11, f = C-8–C-12

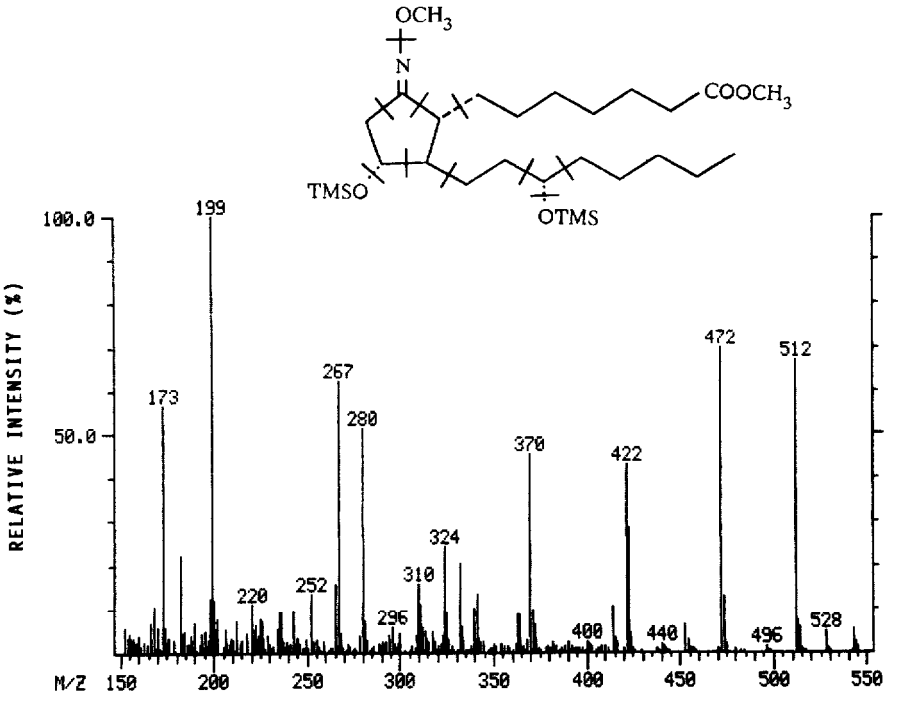


Fig 2 Electron-impact mass spectrum of fragment ions of the methyl ester–methoxime—trimethylsilyl ether derivate of 13,14-dihydro-$PGE_1$ ($H_2PGE_1$; second oxime isomer) The thin lines indicate the sites of fragmentation Two isomer peaks were detected by gas chromatography corresponding to the *syn*- and *anti*-isomers of the methoxime group at C-9

## DISCUSSION

The HPLC method described here is suitable for the separation and the sensitive and specific detection of $PGE_1$ and its primary metabolites. The sensitivity and specificity for the determination of the unlabelled compounds can be further increased by radioimmunological analysis [14,15] of the HPLC eluate fractions.

This method should be applicable to kinetic studies in patients receiving $PGE_1$ infusions for treatment of arterial occlusive disease. In this context, an important aspect of the method is the possibility of detecting and determining specifically $H_2PGE_1$. This metabolite differs from $KPGE_1$ and $KH_2PGE_1$ by its much higher biological activity [7], which for inhibition of human platelet aggregation has been found to exceed even that of the parent compound $PGE_1$ [16]. Preliminary studies [17] have, indeed, demonstrated the occurrence of low concentrations of immunoreactive material coeluting with standard $H_2PGE_1$ in the HPLC system used in the blood of patients receiving intravenous $PGE_1$ infusions. The method described here might be particularly suitable for investigations of the kinetics of this interesting metabolite, and also the therapeutic effects of $PGE_1$ administered intravenously [18].

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