Journal of Chromatography, 529 (1990) 21–32
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5293

Determination of prostaglandins in human seminal fluid by solid-phase extraction, pyridinium dichromate derivatization and high-performance liquid chromatography

JO DOEHL[a,*] and TYGE GREIBROKK

Department of Chemistry, University of Oslo, P.O. Box 1033, Blindern, 0315 Oslo 3 (Norway)

(First received July 21st, 1989; revised manuscript received February 7th, 1990)

ABSTRACT

Prostaglandins of the E type (PGEs) in human seminal fluid have been determined by reversed-phase high-performance chromatography on a C_{18} column and ultraviolet detection at 230 nm after solid-phase extraction (C_{18}) and oxidation to the corresponding 15-oxoprostaglandin derivatives by pyridinium dichromate in acetonitrile. Under optimized conditions, PGEs from 10-ml seminal samples were extracted into 4 ml of methyl formate with high recoveries (estimated at greater than 95%) and subsequently separated under mild chromatographic conditions (0.5 m*M* formic acid–acetonitrile, apparent pH 3.8). Comparable analytical sensitivities were obtained with detection at 230 nm with a conventional deuterium lamp spectrophotometer and a photometer equipped with a cadmium emission source, while with a diode-array spectrophotometer, signal-to-noise ratios were reduced with factors between 4.4 and 3.1, depending on the spectral bandwidth of the instrument. Theoretical aspects of signal-to-noise optimization of ultraviolet detectors are discussed. The stability of dilute standard solutions of PGE_2 and PGD_2 was measured, showing solutions in dichloromethane at 20°C to be as stable as acetonitrile solutions at 5°C over a period of thirty days. Absolute ethanol and acetonitrile were equally suited as solvents.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a well established technique for the separation of compounds within the bio-

[a]Present address: Center for Industrial Research, P.O. Box 124, Blindern, 0314 Oslo 3, Norway.

0378-4347/90/$03.50

chemically important class of prostaglandins (PGs)[a]. Although satisfactory chromatographic conditions for many PGs are readily obtained, detection is not simple and has led to the development of several methods, many of which have recently been reviewed [1]. We have previously reported a UV derivatization procedure in which E-type PGs (PGEs) are selectively oxidized to their UV-absorbing 15-oxo derivatives by pyridinium dichromate (PDC) in acetonitrile [1–3]. Prior to derivatization, PGs can be extracted from biological samples using a variety of procedures. By employing reversed-phase solid-phase extraction columns, PGs can be recovered in small volumes of organic solvents in high yields (greater than 95%) after sequentially removing both more polar and less polar components of the sample matrix on a single column [4]. The aims of the present study were to demonstrate the application of the PDC derivatization to the determination of PGs in human seminal fluid and to establish optimum conditions for the solid-phase extraction, separation and detection of PGs in this matrix. Reduced PGE levels in semen have previously been claimed to correlate with reproduction anomalies such as reduced fertility [5–8] and polyzoospermia [9].

EXPERIMENTAL

Chemicals

All PGs were obtained from Sigma (St. Louis, MO, U.S.A.), PDC (98%) from Aldrich (Milwaukee, WI, U.S.A.), formic acid (p.a.) and methyl formate (p.a.) from Merck (Darmstadt, F.R.G.). Methyl formate and ethyl acetate were redistilled prior to use. Acetonitrile, ethyl acetate, dichloromethane and hexane were HPLC grade from Rathburn (Walkerburn, U.K.). Ethanol (99.9% and 96%) was obtained from Vinmonopolet (Oslo, Norway). Deionized water was distilled once. PG standards were dissolved in acetonitrile ($2.8 \cdot 10^{-4}$ *M*) and stored at $-20°C$.

Chromatography

Sep-Pak™ C_{18} extraction columns were obtained from Waters. The HPLC instrumentation consisted of a solvent-delivery system (Waters Model 590), a valve loop injector (Waters U6K) connected to a C_{18} column [Brownlee MPLC™, 220 mm × 2.1 mm I.D., 5 μm C_{18} silica (Spheri-5) or Perkin-Elmer, 33 mm × 4.6 mm I.D., 3 μm C_{18} silica (Pecosphere)] and a UV detector [Shimadzu SPD 2AM spectrophotometer (conventional geometry), Waters EWM Cd lamp photometer or Hewlett-Packard 1040A spectrophotometer (diode-array)]. The conventional spectrophotometer had a spectral resolution of 7

[a]In this paper, prostaglandins are collectively referred to as PGs, ($\Delta^{13,14}$)-prostaglandins as PG_1 and ($\Delta^{13,14}$, $\Delta^{5,6}$)-prostaglandins as PG_2; PG without a subscript denotes PG_1 *and* PG_2 (PGE = PGE_1 *and* PGE_2).

nm and adjustable time-constants of 0.25, 0.55 and 1.5 s. The biological samples were separated on the 220 mm×2.1 mm I.D. column, whereas the short 33 mm×4.6 mm I.D. column was used for all stability and recovery measurements. Water, acetonitrile (HPLC grade S) and formic acid were used as the mobile phase.

Derivatization of PGs

PGs were oxidized by PDC in acetonitrile, diluted with water and extracted with ethyl acetate to remove excess reagent as previously described [2,3].

Sample preparation and solid-phase extraction

Human seminal fluid from a healthy, fertile donor was immediately frozen and stored at −20°C until analysis. Samples were diluted to 1.0 ml and adjusted to pH 3.5 with aqueous formic acid. Small samples (<50 μl seminal fluid) were directly transferred to the extraction column after dilution, while larger samples were centrifuged prior to extraction. Solid-phase extraction on Sep-Pak™ C_{18} extraction columns followed with minor changes the general outline of Powell [4]. (1) Application of 1 ml of acidified sample; (2) elution of polar components with 20 ml of ethanol–water (15:85, v/v); (3) removal of ethanol with 20 ml of water; (4) mechanical removal of water and elution of lipophilic components with 20 ml of hexane: PGs are retained on the column; (5) elution of PGs with 4 ml of methyl formate.

Extraction columns were prepared and regenerated by passing 20 ml of ethanol and 20 ml of water through them prior to use.

RESULTS AND DISCUSSION

Solid-phase extraction

Several solvents have been recommended for the elution of PGs from C_{18} extraction columns, among them acetonitrile, methanol, diethyl ether and methyl formate [4]. Fig. 1 shows recovery data for standard samples of PGE_2 and PGD_2 using different solvents and elution procedures. PGD_2 was added as a sensitive probe for unsuitable analytical PG conditions since it is isomeric with, but less stable than PGE_2, as will be shown below. Clearly, methyl formate was by far the most efficient solvent, resulting in recoveries of PGE_2 and PGD_2 of 97±4 and 91±4%, respectively, in 10 ml of solvent ($n=4$). Identical volumes of other solvents (acetonitrile, acetone, diethyl ether or methanol) all gave recoveries of less than 90% (PGE_2) and 75% (PGD_2). Reducing the volume of ethanol–water (15:85, v/v) in the procedure from 20 to 1 ml, or acidifying this solvent to an apparent pH 3.3, did not significantly influence the recovery ($p<0.05$). Pure hexane did not elute PGs and could therefore safely replace petroleum ether in the original procedure [4]. Even after addition of a

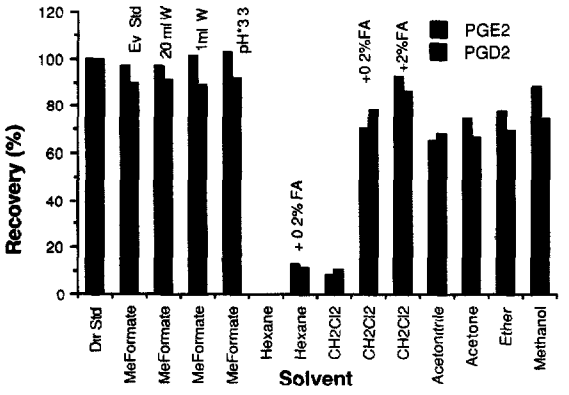

Fig. 1. Recoveries of PGD_2 and PGE_2 (1 μg) from C_{18} extraction columns eluted with different solvents, measured by RP-HPLC (area measurement, mean of four injections, UV detection at 190 nm). Presented data are the mean of four experiments. Abbreviations: Dir Std = direct standard injection of mobile phase solutions of PGs (external standard); Ev Std = evaporated 10 ml methyl formate standard solutions of PGs reconstituted without passing the solution through the extraction column; 20 ml W = elution with 20 ml of ethanol–water (15:85, v/v) included in the procedure; 1 ml W = elution with 1 ml of ethanol–water (15:85, v/v) included in the procedure; pH* 3.3 = elution with 10 ml of ethanol–water (15:85, v/v) acidified to pH* 3.3 included in the procedure; +0.2% FA = formic acid (0.2%) added to the solvent; +2% FA = formic acid (2%) added to the solvent.

polar modifier (i.e. formic acid), recoveries were less than 15%. By adding 2% (v/v) of formic acid to dichloromethane, recoveries were improved (90% PGE_2, 85% PGD_2), but were still less than those obtained with methyl formate.

Recoveries of PGs are often measured by radioactivity regain measurements of labelled standards. The data in Fig. 1, however, were obtained by reconstitution of extracted standards in the mobile phase and measurement of peak areas by RP-HPLC against external standards. In contrast to the former method, the integrity of the recovered compound can be monitored and not only the unspecific recovery of labelled substituents. Thus, it was possible to observe that the reduced recovery of PGD_2 compared with PGE_2 was caused by degradation during the solvent evaporation and not by retention on the extraction column, since reduced amounts of PGD_2 were also recovered from methyl formate solutions that were not passed through the column (Fig. 1). In our experience, even redistilled methyl formate contained higher-boiling contaminants, possibly of a polar nature (e.g. formic acid), which accumulated towards the end of the drying process, where some gentle heating usually had to be applied to remove all the solvent reasonably quickly. It is therefore important to keep the solvent volume at a minimum. A 4-ml volume of methyl formate was found to elute 100 ng of PGE_2 and PGE_1 quantitatively from the

extraction column, substantially less than 10 ml as was previously recommended [4].

Stability of PG standards

Quantitative measurements of PGs are dependent on the availability of PG standards with reliable concentration values. PGD and PGE both contain a labile β-ketol function, which is easily dehydrated, particularly in protic solvents. The influence of the solvent on the stability is demonstrated in Figs. 2 and 3. The concentrations of PGD_2 and PGE_2 in standard solutions (2 μg/ml) were monitored by RP-HPLC over thirty days. All solutions were kept at ambient temperature (20±2°C) in ordinary laboratory daylight except for one acetonitrile solution, which was kept at 5°C to measure the effect of temperature. An acetonitrile solution of the two PGs stored at −20°C was used as

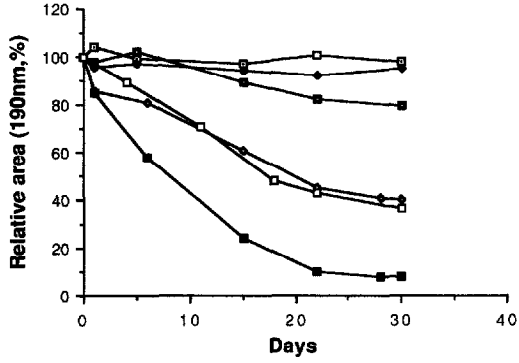

Fig. 2. Stability of PGD_2 (2 μg/ml) in different solvents. (⊡) Acetonitrile at 5°C; (♦) dichloromethane; (■) ethyl acetate; (◇) acetonitrile at 20°C; (■) ethanol (96%); (□) ethanol (absolute).

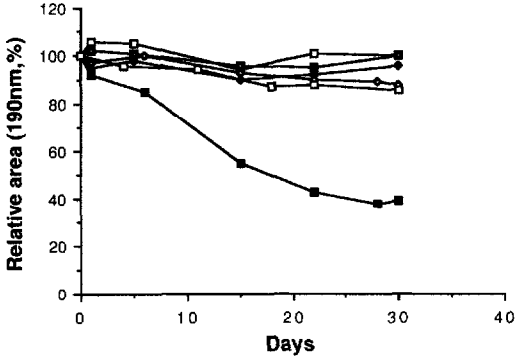

Fig. 3. Stability of PGE_2 (2 μg/ml) in different solvents. Symbols as in Fig. 2.

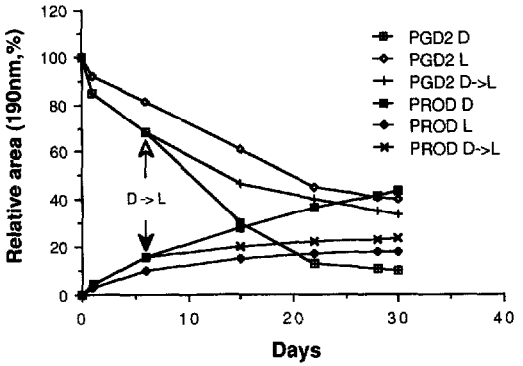

Fig. 4. Stability of PGD_2 under different light conditions. D, Stored in the dark; L, exposed to laboratory light; D→L, conditions changed from dark to light after six days; PGD2, PGD_2; PROD, unidentified degradation product of PGD_2.

external standard. As expected, the degradation rate increased with solvent polarity. This is best illustrated by the degradation profiles of PGD_2, the less stable of the two compounds (Fig. 2). Whereas the loss of PGD_2 in dichloromethane was less than 5%, the losses were 20% in acetonitrile and absolute ethanol and more than 90% in ordinary rectified-grade (96%) ethanol. Clearly, the water content of the solvents should be minimized. It should be noted that the stabilities of PGD_2 and PGE_2 were not significantly different in dichloromethane at 20°C and acetonitrile at 5°C within the investigated period of thirty days. Dichloromethane can therefore be recommended as a solvent for PGs in low concentrations. Solutions in acetonitrile and absolute ethanol had comparable stabilities and are well suited as solvents, if kept cold (5°C).

In several experiments it was observed that acetonitrile solutions of PGD_2 stored in the dark were less stable than identical solutions exposed to laboratory daylight. Fig. 4 illustrates the degradation of PGD_2 and the appearance of one unidentified degradation product in two identical solutions, with and without exposure to light. After six days in the absence of light, one solution was divided into two, and one half was exposed to light. The data reveal a significant reduction in PGD_2 degradation on exposure to light in this solvent, a phenomenon that remains to be explained.

Determination of PGEs in human seminal fluid

The RP-HPLC separation of oxidized PGs from human seminal fluid is shown in Fig. 5. After solid-phase extraction and removal of the solvent under a stream of nitrogen, the sample was derivatized by PDC prior to separation by HPLC as previously described [2,3]. With reference to Fig. 5, the six major peaks were identified as 15-oxo-19-hydroxy-PGE_2 (A), dioxo-PGE_2 (B), 15-oxo-19-hydroxy-PGE_1 (C), dioxo-PGE_1 (D), 15-oxo-PGE_2 (E) and 15-oxo-

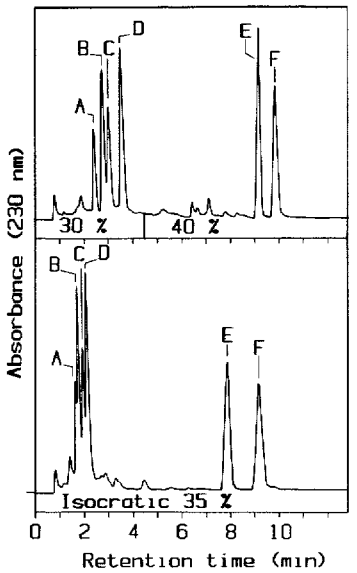

Fig. 5. RP-HPLC separation of PGs in human seminal fluid derivatized by PDC. Column, Brownlee 5-μm C_{18} silica (220 mm×2.1 mm I.D.); mobile phase, acetonitrile–0.5 m*M* formic acid (aqueous) (35:65, lower half; 30:70 to 40:60, upper half) at 0.4 ml/min; detection, cadmium lamp UV photometer at 228.6 nm. Peaks: A = 15-oxo-19-hydroxy-PGE_2; B = dioxo-PGE_2; C = 15-oxo-19-hydroxy-PGE_1; D = dioxo-PGE_1; E = 15-oxo-PGE_2; F = 15-oxo-PGE_1.

PGE_1 (F) by their UV absorption spectra and direct chemical ionization mass spectra (DCI-MS). The dioxo-PGs are most probably the 15,19-dioxo isomers, since the hydroxyl group at C-11 was only oxidized to a small extent with PGE_1 and PGE_2 [2,3]. With UV spectroscopy, the 15-oxo-PG_1 and -PG_2 compounds could readily be distinguished because of the pronounced absorption of the latter at 190 nm [3]. DCI mass spectra resulted in prominent $(MH - n \cdot H_2O)^+$ ions, the number of water losses reflecting the number of hydroxyl and/or ketone functions in the molecule [1,10]. The resolution of the oxidized 19-hydroxy-PGE_1 and -PGE_2, which was poor under the isocratic conditions suitable for the separation of 15-oxo-PGE_1 and -PGE_2, was considerably improved by a step gradient (Fig. 5). Baseline resolution of all oxidized 19-hydroxy-PGEs required weak initial eluting conditions owing to a selectivity change between compounds B and C at 25% acetonitrile (Fig. 6). The formation of doubly oxidized derivatives of 19-hydroxy-PGE was reduced by reducing the PDC concentration, reaction temperature and reaction time, but even under these circumstances the formation of significant amounts (greater than 10%) could not be avoided. The PDC derivatization of these compounds will therefore be restricted to qualitative purposes. The selectivity of the PGE oxidation, however, permitted the quantitation of PGE_1 and PGE_2 in small samples of

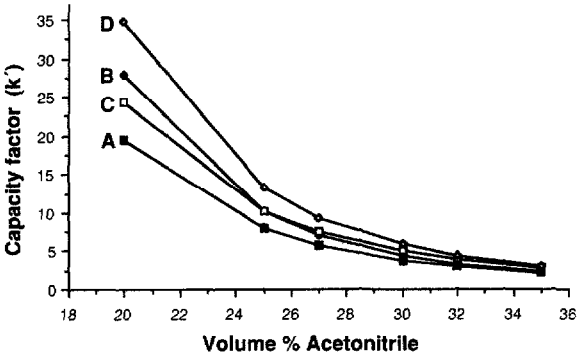

Fig. 6. Capacity factors of 19-hydroxy-PGs in human seminal fluid derivatized by PDC: (A) 15-oxo-19-hydroxy-PGE_2; (B) dioxo-PGE_2; (C) 15-oxo-19-hydroxy-PGE_1; (D) dioxo-PGE_1.

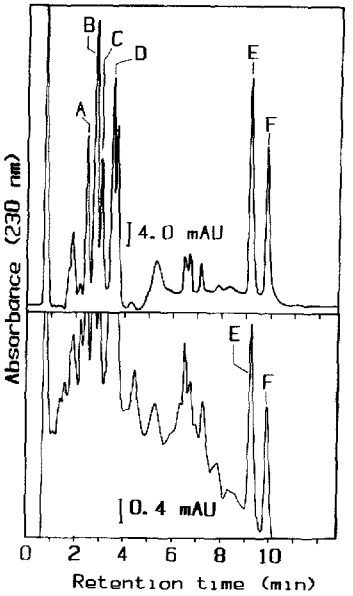

Fig. 7. RP-HPLC separation of PGs in small volumes of human seminal fluid after C_{18} solid-phase extraction and PDC derivatization. Total sample volume, 10 μl (upper half), 1 μl (lower half); one third of sample injected after reconstitution in the mobile phase. Other conditions and peaks as in Fig. 5.

seminal fluid (1–10 μl), as shown in Fig. 7. Over a period of fourteen days, the PGE_1 and PGE_2 concentrations in the semen from one donor were determined on four occasions as 38 ± 3 and 25 ± 2 μg/ml, respectively ($n = 16$, external standard calibration). Day-to-day variations were not statistically significant ($p < 0.05$). These observations are in agreement with previously reported total

PGE average concentrations in the semen of fertile men, which were measured in the range from 44 to 73 μg/ml [9].

Resolution dependence on pH

In the literature on separation of PGs as free acids, RP-HPLC is usually performed with mobile phases containing 2.5–170 m*M* aqueous buffers or acids as the most polar component [11–19]. In our experience the concentration of buffers, and in particular acids, should be minimized, since low pH values shorten the lifetime of the stationary phase, and buffers may increase pump wear. In addition, high concentrations of buffers and acids reduce the UV transparency of the mobile phase. This is particularly important when detecting non-derivatized PGs at low UV wavelengths (190 nm). Columns exposed to acetonitrile–5–10 m*M* aqueous formic acid (65:35, v/v) [apparent pH (pH*) <3.2] over several months, lost significant amounts of their C_{18} stationary phase, as manifested by reduced capacity factors, reduced pressure drop, loss of selectivity as well as degradation of efficiency and peak symmetry. As illustrated in Fig. 8, the resolution of a critical triplet of PGs (PGE_2, PGE_1 and PGD_2, in order of elution) remains constant in the concentration range 0.5–5 m*M* formic acid in the aqueous component of the mobile phase. We, therefore, recommend acetonitrile–0.5 m*M* formic acid (aqueous) (pH* 3.8) as a suitable mobile phase for PGs.

Detector sensitivity

Table I compares the analytical sensitivities obtained with 15-oxo-PGE_2 (0.1 μg) using different detectors. The measured signal-to-noise (S/N) ratios indicated a sensitivity of the conventional spectrophotometer (Shimadzu SPD

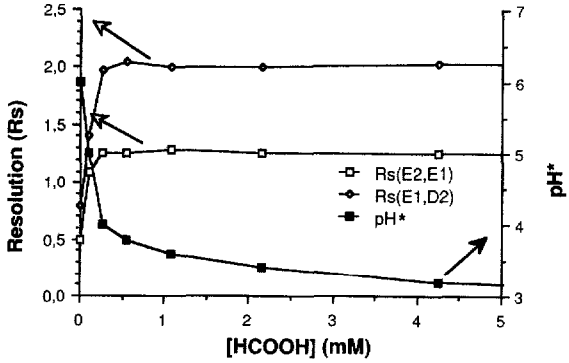

Fig. 8. Left abscissa: resolution of PGE_2/PGE_1 [Rs(E2,E1)] and PGE_1/PGD_2 [Rs(E1,D2)] as a function of formic acid concentration in the mobile phase, acetonitrile-formic acid (aqueous) (35:65, v/v). Right abscissa: apparent pH (pH*) as a function of formic acid concentration in the mobile phase, acetonitrile-formic acid (aqueous) (35:65, v/v).

TABLE I

SIGNAL, NOISE AND SIGNAL-TO-NOISE RATIOS OBTAINED WITH THREE DIFFERENT UV DETECTORS

Identical samples (ca. 100 ng of 15-oxo-PGE_2) were used; the detection wavelength was 230 nm.

Detector	Conditions	Signal (10^{-3} A.U.)	Noise (10^{-5} A.U.)	Signal-to-noise (10^3)
Waters	+ Pulse dampener	64	2.4	2.7
EWM (Cd)	− Pulse dampener	64	4.6	1.4
Shimadzu	Time-constant 0.55 s	64	1.7	3.8
SPD 2AM	Time-constant 0.25 s	68	2.6	2.6
HP 1040A	Bandwidth 8 nm	40	6.8	0.59
	Bandwidth 44 nm	33	4.0	0.83

2AM) that was equal to or better than the values measured with the Cd lamp photometer (Waters EWM). This is in contrast to our previous findings [3] and is due to improvements in detector technology, which have reduced the noise levels by an order of magnitude in some UV spectrophotometers over the last decade. With the column used (33 mm×4.6 mm I.D., 3-μm C_{18}), the 15-oxo-PGE_2 peak had a standard deviation (σ) of 2.2 s. A time-constant larger than the spectrophotometer minimum setting (τ=0.25 s) should therefore be avoided, using the standard 10% criterion to maintain peak shape. It should be mentioned that a pulse dampener had to be inserted in front of the Cd photometer, since this detector was particularly sensitive to pump noise (Table I).

To get comparable values from the diode-array detector (DAD, HP 1040A), optimization of measurement parameters had to be performed. With a DAD the resolution can be varied in both the time domain (acquisition rate) and the spectral domain (spectral bandwidth). Decreasing the resolution will decrease the noise, since from statistical theory, the standard error of the mean of n measurements (i.e. noise) is proportional to $(n)^{-0.5}$, provided the standard error distribution is Gaussian. Increasing the number of measurements of each averaged data point is equivalent to decreasing the resolution. Thus, by trading off resolution in either dimension, a total improvement of the S/N ratio by a factor of 3–4 can be expected, the effect of each dimension being multiplicative [20]. In the time domain, the acquisition rate in our measurements was fixed at a minimum of twenty data points per peakwidth (5σ) to maintain peak shape for quantitation. With PGE_2 on the short column this corresponded to 1.8 Hz. In the spectral domain, the S/N ratio was increased by a factor of 1.7 by increasing the spectral bandwidth from 4 to 40 nm (Fig. 9). This is in contrast to statistical theory, from which a gain in the S/N ratio of 2.5 times might be expected, as illustrated in Fig. 9. Signal values in Fig. 9

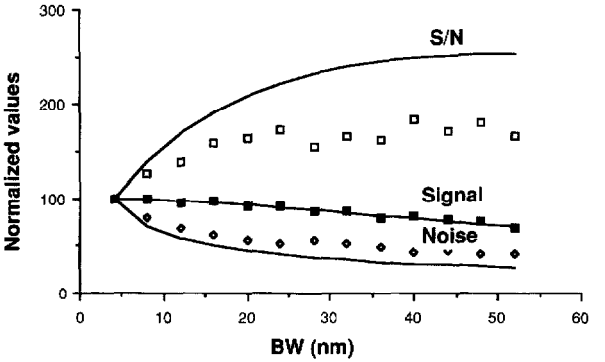

Fig. 9. Signal, noise and signal-to-noise ratios on a diode-array detector (solid curves) as well as measured values (points) for HP 1040A as a function of spectral bandwidth (BW). A.U. values are for 15-oxo-PGE_2 (0.1 μg) at a centre wavelength of 230 nm.

were calculated from the UV spectrum of PGE_2 integrated over the spectral bandwidth (BW) and were in accordance with measured values. Noise was calculated assuming proportionality with $(BW)^{-0.5}$ as stated above. The discrepancy between calculated and measured noise values indicates that a significant part of the noise is not caused by independent events within each diode element, but by events affecting several diodes of the array simultaneously. Possible origins of such covariance are variations in lamp intensity, refractive phenomena in the light path and certain electronic variations in the detector circuitry. Similar conclusions can be drawn from previously reported data, which, however, were not commented by the authors [20].

From Table I it can be concluded that the DAD was 3.1–4.4 times less sensitive than the conventional spectrophotometer, depending on the chosen spectral resolution of the former (8–44 nm). The spectral resolution of the latter was fixed (8 nm).

REFERENCES

1 J. Doehl and T. Greibrokk, J. Chromatogr., 477 (1989) 345.
2 J. Doehl and T. Greibrokk, J. Chromatogr., 282 (1983) 435.
3 J. Doehl and T. Greibrokk, J. Chromatogr., 349 (1985) 431.
4 W.S. Powell, Methods Enzymol., 86 (1982) 467.
5 D.F. Hawkins, in P.W. Ramwell and J.E. Shaw (Editors), Prostaglandin Symposium of the Worcester Foundation, Interscience, New York, 1968, pp. 1–10.
6 M. Bygdeman, B. Fredricsson, K. Svanborg and B. Samuelsson, Fertility Sterility, 21 (1970) 622.
7 H.C. Brummer and A. Gillespie, Clin. Endocrinol., 1 (1972) 363.
8 J.G. Collier, R.J. Flower and S.L. Stanton, Fertility Sterility, 26 (1975) 868.
9 E. Bendvold, C. Gottlieb, K. Svanborg, M. Bygdeman and P. Eneroth, Int. J. Androl., 10 (1987) 463.

10 H.Y. Kim, J.A. Yergey and N. Salem, Jr., J. Chromatogr., 394 (1987) 155.
11 K. Nagayo and N. Mizuno, J. Chromatogr., 178 (1979) 347.
12 D.M. Desiderio, M.D. Cunningham and J.A. Trimble, J. Liq. Chromatogr., 4 (1981) 1261.
13 A. Terragno, R. Rydzik and N.A. Terragno, Prostaglandins, 21 (1981) 101.
14 R. Freixa, J. Casas, J. Rosello and E. Gelpi, J. High Resolut. Chromatogr. Chromatogr. Commun., 7 (1984) 156.
15 R. Rydzik, A. Terragno and R. Tackett, J. Chromatogr., 308 (1984) 31.
16 C. Surrenti, O.E. Ricci, A. Casini, L. Ventura and S. Nieri, J. Liq. Chromatogr., 7 (1984) 2409.
17 K. Yamamura and T. Yotsuyanagi, J. Chromatogr., 303 (1984) 165.
18 C.A. Herman, M. Hamberg and E. Granstrom, J. Chromatogr., 394 (1987) 353.
19 E.H. Oliw, J. Chromatogr., 421 (1987) 117.
20 S.A. George and H. Elgass, Int. Lab., September (1984) 42.