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QUANTITATIVE ANALYSIS OF PROSTACYCLIN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Prostacyclin (prostaglandin (PG) I_2) was discovered by J. R. Vane and his co-workers in 1976 [1]. It appeared to be the strongest inhibitor of platelet aggregation described so far [1]. Also, PGI_2 was further characterized as a potent vasodilator and cytoprotector [1, 2]. These properties make it an important candidate for therapeutic use. Unfortunately, PGI_2 is chemically extremely unstable. In acid or neutral aqueous conditions, its cyclic enolether unit is hydrolyzed within a few minutes into 6-keto-$PGF_{1\alpha}$ [1, 3, 4]. Although under unhydrous conditions PGI_2 Na-salt is stable in crystalline form, determination of the chemical purity of prostacyclin samples is obligatory for investigators studying the physiological activity of this compound.

The chemical unstability of PGI_2 gives rise to serious problems in its analysis. In solving these problems, G. T. Hill suggested the use of high performance liquid chromatographic method on reverse phase columns [5]. M. A. Wynalda, F. N. Lincoln and F. A. Fitzpatrick found an excellent mobile phase for eluting PGI_2 Na-salt and its impurities. It consists of acetonitrile and water buffered at pH 9.3 with boric acid and sodium borate [6]. They used the external standard method that requires full-loop injections and regular peak shapes. The external standard method requires, before each series of sample analysis, injection of PGI_2 Na-salt with high purity for comparison. This gives once more rise to some problems due to the unstability of PGI_2 Na-salt. Besides, the precision of the method is low [6]. Such a situation suggests the need for the development of an internal standard method for analyzing PGI_2 Na-salt.

Two methods were tested. In the first method one internal standard and specially purified PGI_2 Na-salt are used for calibration and in the second one two internal standards plus technical grade PGI_2 Na-salt are employed.

Materials and methods

Apparatus. A DuPont HPLC system N 8845 with a Rheodyne Model 7125 Syringe Loading Sample Injector (maximum sample size 50 µl). A spectrophotometric detector was set at 205 nm and 0.64 AUFS. A recorder operated at 10 cm/h.

Reagents. Boric acid, sodium borate and sodium hydroxide (Reakhim, USSR) were used without purification. Acetonitrile (Reakhim) was redistilled. Bidistilled water was used. PGI_2 Na-salt (99% by HPLC, mp. 164—166°) was prepared according to [7–10] and crystallized twice from water-acetonitrile (0.5:15). 6-keto-$PGF_{1\alpha}$ methyl ester was prepared in different batches according to [7]. Ester moiety was hydrolyzed by a 1N·NaOH solution in methanol. Biosynthetic PGE_1 (mp. 114—116°)

used also as an internal standard was tested to be at least 99% pure by HPLC [11].

Chromatographic conditions. Chromatography was performed at ambient temperature on a 4.0×220 mm Zorbax ODS column or 4.6×250 mm column prepacked with a Zorbax ODS. The columns were eluated with acetonitrile-water (85—87% of water, buffered at pH 9.3 with 0.01 M boric acid and 0.006 M sodium borate) at a flow rate of 0.8—1.0 ml/min.

Direct analysis with PGE_1 as an internal standard. Method A. In order to obtain the calibration curve, 5 different quantities of pure PGI_2 Na-salt in the range of 1.0—3.5 mg were weighed (W_I) and the precise amounts (1.0—3.5 mg) of PGE_1 (W_E) were added; the samples were dissolved in 300 µl of water buffered at pH 10.1 with 0.013 M sodium borate and 0.023 M sodium hydroxide at 4°. In each case, 5 µl of the sample was injected and the rest was kept at 4° between reinjections. Each sample injection was performed twice.

Analyses with PGE_1 and 6-keto-$PGF_{1\alpha}$ as internal standards. Method B. In order to obtain the calibration curve, 11 different quantities of PGI_2 Na-salt in the range of 1.0—3.5 mg were weighed and the precise amounts (1.0—3.5 mg) of PGE_1 (W_E) were added. An aqueous solution of 6-keto-$PGF_{1\alpha}$ was prepared in a concentration of 10 mg/ml, buffered at pH 10.1 with 0.013 M sodium borate and 0.023 M sodium hydroxide and kept at 4° before using. Thereupon, 8—100 µl of the 6-keto-$PGF_{1\alpha}$ solution (the weight of 6-keto-$PGF_{1\alpha}$ (W_F)) was added to the weighed samples of PGI_2 Na-salt. The same buffer was added till the total volume of the sample of 300 µl. In each case, 5 µl of the sample was injected and the rest was kept at 4° between reinjections. Each sample injection was performed twice. Subsequently, PGI_2 Na-salt was converted into 6-keto-$PGF_{1\alpha}$ by acidifying the samples with acetic acid up to pH 5—6. Then, 0.1 N sodium hydroxide was added at 4° up to pH 8—9 and 15 µl of the sample was injected. The samples were stored and reinjected as described above.

Calculations. The peak areas were calculated by «Height×Width at Half Height» technique [12].

Response factor of PGI_2 Na-salt versus PGE_1

$$f=\frac{A_I W_E}{A_E W_I} \qquad (1)$$

where A_I and A_E are the peak areas of PGI_2 Na-salt and PGE_1, W_I and W_E are their weights, respectively.

Corrected PGE_1 peak area

$$A_E^{corr}=A_E-A_I\times k \qquad (2)$$

where k is the area fraction of interfering impurity in PGI_2 Na-salt.

Formulas analogous to (1) and (2) may be used in calculations for 6-keto-$PGF_{1\alpha}$ when PGI_2 Na-salt contains 6-keto-$PGF_{1\alpha}$.

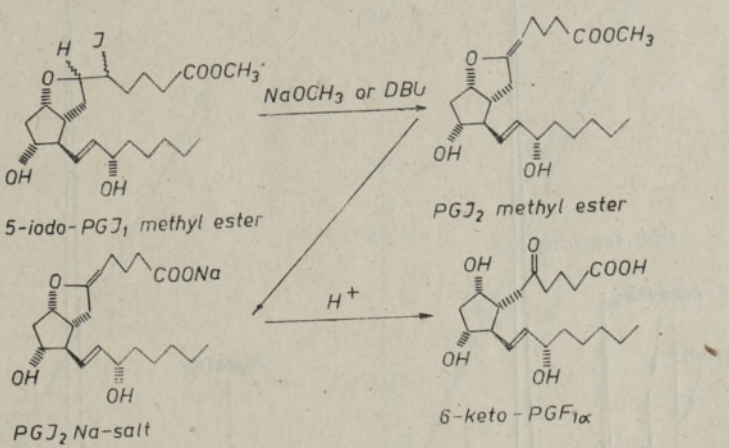
Real weight of PGI_2 Na-salt (the difference in molecular masses of 6-keto-$PGF_{1\alpha}$ and PGI_2 Na-salt is neglected)

$$W_I^{real}=\left(\frac{R''_A}{R'_A}-1\right)W_F \qquad (3)$$

where R'_A and R''_A are the ratios of peak areas of 6-keto-$PGF_{1\alpha}$ to these of PGE_1 before and after acidification, respectively, W_F is the weight of 6-keto-$PGF_{1\alpha}$.

Results and discussion

PGI_2 Na-salt is synthesized from 5-iodo-PGI_1 methyl ester according to the following scheme:



The main impurities of PGI_2 Na-salt (technical grade) are 6-keto-$PGF_{1\alpha}$, an unidentified compound in an amount of 1—3.5% of PGI_2 Na-salt and inorganic salts (NaOH, NaI).

The internal standard method requires that the standard should eluate near the peaks of interest and should be of high purity and chemically stable. We found that PGE_1 meets these requirements. It has an acceptable retention time under the chromatographic conditions used. The capacity factor k', measured on a Zorbax ODS column for 6-keto-$PGF_{1\alpha}$, PGE_1, and PGI_2 Na-salt, was 0.9, 3.4 and 9.0, respectively (Fig. 1). The fact that PGE_1 eluates between 6-keto-$PGF_{1\alpha}$ and PGI_2 Na-salt is also of importance. The purity of the crystalline PGE_1 (99% by HPLC) meets the analytical requirements.

It is well known that under basic conditions PGE_1 converts into PGB_1 via PGA_1 [13, 14]. By examining UV-spectra of PGE_1 at ambient temperature we found that at pH 9.3 less than 1% of PGE_1 is degradated into PGA_1 ($\lambda_{max}=217$ nm; $\varepsilon=10830$) [15] during 1 h. At pH 10.1 1.4% of PGE_1 was converted into PGA_1 in 30 min. The conversion of PGA_1 into PGB_1 ($\lambda_{max}=278$ nm; $\varepsilon=26800$) [16] proceeds much slower. Only 0.7% of PGE_1 can be converted into PGB_1 in 18 hs at pH 10.1. Also, by HPLC two injections from one sample under the above conditions were coincident with a precision of 1%. No trace amounts of PGA_1 or PGB_1 were detected.

Calibration of PGI_2 Na-salt versus PGE_1 was performed by two different methods. In the first method (method A) the twice recrystallized PGI_2 Na-salt (Fig. 2) was used. The real peak areas of the weighed amounts of PGI_2 Na-salt and PGE_1 were measured. An assumption was made that the response of the detector to PGI_2 Na-salt and PGE_1 is linear to their amount in the investigated region. From the calibration curve obtained (Fig. 3, *a*) the response factor f_A of PGI_2 Na-salt versus PGE_1 on the UV-detector at 205 nm was calculated and found to be 3.65 ± 0.15 at a confidence level of 95%.

By the second method (method B), the peak areas of 6-keto-$PGF_{1\alpha}$ (A_F) and PGI_2 Na-salt (A_I) were measured and the peak area of PGE_1 was calculated according to formula (2). For each sample the ratio of the peak areas of 6-keto-$PGF_{1\alpha}$ to the peak area of PGE_1 (R'_A) was calculated. Analogously, these ratios (R''_A) were determined after acid-

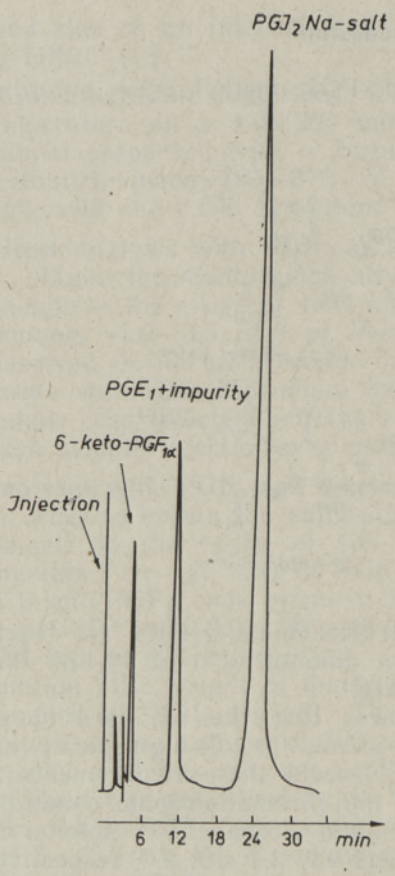


Fig. 1. Chromatogram of a mixture of 6-keto-$PGF_{1\alpha}$ and PGE_1 and PGI_2 Na-salt (technical grade) used for calibration in method B. Chromatography was performed on a Zorbax ODS (4.6×250 mm) column using acetonitrile-water as mobile phase (13:87, v/v), buffered at pH 9.3 with 0.01 M boric acid and 0.006 M sodium borate. The flow rate was 1.0 ml/min. The detector was set at 205 nm and 0.64 AUFS. The recorder operated at 10 cm/h.

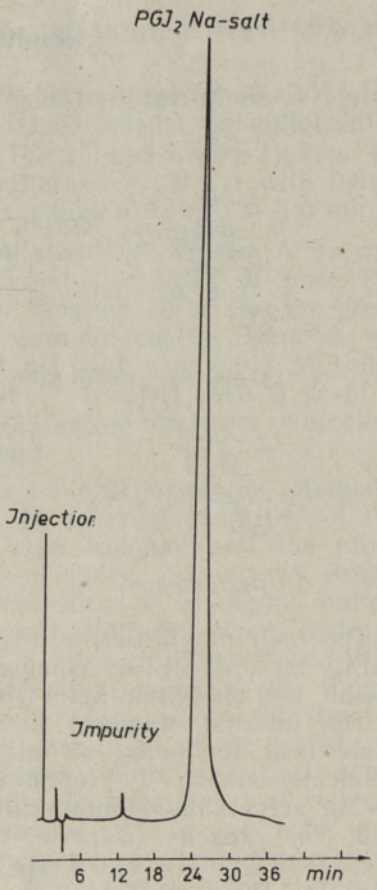


Fig. 2. Chromatogram of PGI_2 Na-salt (standard grade) used for calibration in method A. Chromatographic conditions are as in Fig. 1.

ifying the samples. From the increase in the peak areas ratio the amount of PGI_2 Na-salt (W_I) in the sample was calculated according to formula (3). By method B the response factor of PGI_2 Na-salt versus PGE_1 (f_B) was found to be 4.3 ± 1.1 at a confidence level of 95%. The response factor of 6-keto-$PGF_{1\alpha}$ versus PGE_1 was found to be 0.68 ± 0.20 at a confidence level of 95%.

As can be seen from Fig. 3, method A gives a calibration curve which is in accordance with experimental data. Method B, on the contrary, gives a calibration curve, with a great deviation from experimental data. Consequently, method A is superior to method B in the analysis of PGI_2 Na-salt samples. By the statistical treatment of the deviation of experimental points we found that it cannot be subjected to normal distribution. In addition we found that between the experimental points 1—5 and 9—11 (Fig. 3, *b*) (these points correspond to different 6-keto-$PGF_{1\alpha}$ batches) a systematic error occurs. This suggests a

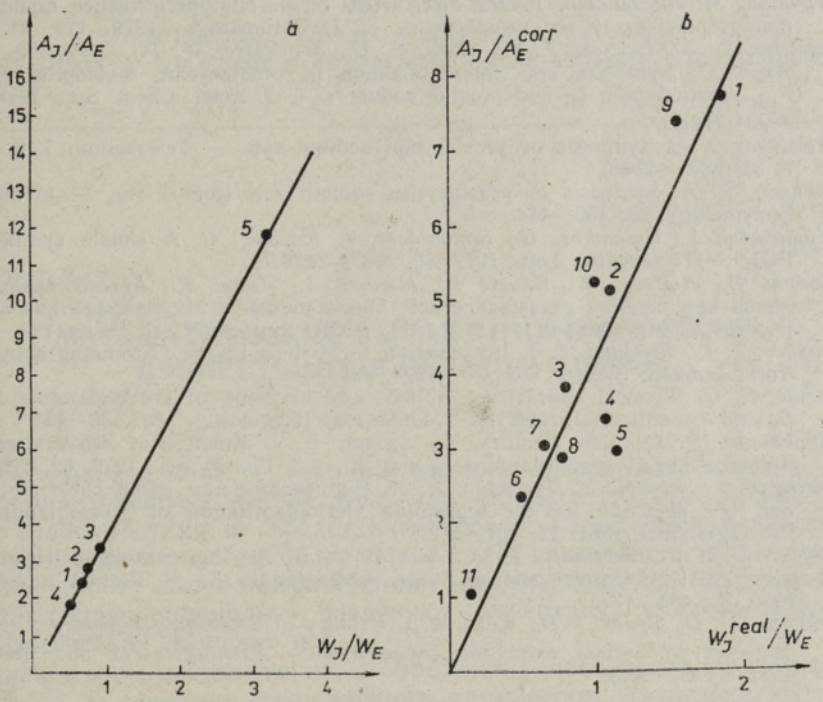


Fig. 3. Calibration curves of PGI_2 Na-salt. *a* — method A, *b* — method B.

conclusion that the purity of 6-keto-$PGF_{1\alpha}$ in different batches is different due to difficulties in the preparation, extraction and purification of this compound [8, 17]. The conditions under which 6-keto-$PGF_{1\alpha}$ can be used for constructing a calibration curve require further study.

Conclusions

1. The internal standard method using PGE_1 as an internal standard for determining the purity of PGI_2 Na-salt was developed.

2. The direct calibration method where a solution of pure PGI_2 Na-salt was used (method A) is superior to the method via 6-keto-$PGF_{1\alpha}$ (method B).

3. PGI_2 Na-salt response factor to PGE_1 at 205 nm is 3.65 with a standard deviation of 0.05. It provides sufficient precision for determining the purity of PGI_2 Na-salt.

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PROSTATSÜKLIINI KVANTITATIIVNE ANALÜÜS KÕRGEFEKTIIVSEL VEDELIKUKROMATOGRAAFIAMEETODIL

On välja töötatud prostatsükliini määramise kvantitatiivne meetod, kasutades sisestandardit. On võrreldud kahte meetodit: A — sisestandardiks oli PGE_1; B — sisestandarditeks olid 6-keto-$PGF_{1\alpha}$ ja PGE_1.

Meetod A andis piisava täpsusega tulemused. 205 nm juures oli PGI_2 detekteerimise tundlikkus 3,65±0,15 korda suurem kui PGE_1-l.

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КОЛИЧЕСТВЕННЫЙ АНАЛИЗ ПРОСТАЦИКЛИНА С ПОМОЩЬЮ МЕТОДА ВЫСОКОЭФФЕКТИВНОЙ ЖИДКОСТНОЙ ХРОМАТОГРАФИИ

Разработан количественный метод определения чистоты натриевой соли простациклина с использованием внутреннего стандарта, в качестве которого служили $ПГЕ_1$ (метод А) и 6-кето-$ПГГ_{1\alpha}$, $ПГЕ_1$ (метод Б). Метод А дал результаты удовлетворительной точности. При 205 нм чувствительность детектирования натриевой соли $ПГI_2$ превышала чувствительность детектирования $ПГЕ_1$ в 3,65±0,15 раза.