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Note

Determination of prostaglandin $F_{2\alpha}$ and 6-oxo-prostaglandin $F_{1\alpha}$ in urine by gas chromatography—positive chemical ionisation-mass spectrometry using stable isotope dilutions with selected ion monitoring

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Isotopically labelled substances are biochemically identical to non-labelled ones, but can be identified separately from naturally occurring ones by means of physical techniques. Isotopically labelled material permits the study of metabolic pathways of naturally occurring substances in vivo and in vitro, aids detection of metabolic disorders and enables the processes of resorption, distribution, storage and excretion to be followed in animals and humans. The development of very sensitive and precise instruments allows the establishment of so-called "definitive methods" and the precise measurement of very low amounts of biological substances using stable isotopes as tracers.

Radioimmunoassay offers exceptional sensitivity and allows the measurement of a large number of samples in a relatively short time, but the method is inherently suspect in terms of specificity. Consider, for example, the influence of unknown cross-reacting substances in body fluids, or of multistage preseparation procedures with a high percentage of losses. Losses in multistage separation methods can be compensated by radioisotopic labelling, but destruction of the molecules during the separation procedure is sometimes disregarded and falsifies the results. For example, cortisol and prednisolone were separated on thin-layer chromatographic (TLC) sheets, the corresponding zones were detected under UV light, and the substances measured by radioimmunoassay. The UV light caused destruction of the cortisol and prednisolone molecules, and neither substance bound to the binding protein (transcortin), but the radioactivity was present in the eluate [1]. Chromatographic procedures tend to give variable results if labile substances are studied or if two sterically different substances result from derivatisation for gas chromatography (GC). Such faults are avoided if the isotopically labelled standard and the substance are both measured with the same detector as is possible with mass spectrometry (MS).

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A sensitive and precise method for measurement of urinary prostaglandins (PG) is presented. Tetradeuterated PGs are used as internal standards and as tracers.

EXPERIMENTAL

Method

The method [2] described for measurement of serum PGs using a negative chemical ionisation (NCI) MS device was adapted for our needs to measure $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$ from urine or from cell incubations with a positive chemical ionisation (PCI) MS device. The method consists of: 20 ml of urine or diluted sample + 50 ng of both PGs in tetradeuterated form + 1.4 ml of 2 *N* HCl → centrifugation → supernatant → sorption of the PGs on CP^{tm} Elut C 18 (Chrompack Cat. No. 19016; Chrompack, Middelburg, The Netherlands) 200 mg of sorbent → washing with 100 ml of water → elution of the PGs with 7 ml of ethyl acetate → sorption of the PGs on normal phase CP^{tm} Elut Si (Chrompack Cat. No. 19010) 500 mg of sorbent → washing with 50 ml of ethyl acetate → extraction of the PGs with 5 ml of methanol → evaporation under nitrogen at room temperature → TLC (Merck Kieselgel 60; Merck, Darmstadt, G.F.R.) solvent system: organic layer of ethyl acetate—acetic acid—isooctane—water (11:2:5:10), standards can be made visible by spraying with phosphomolybdate, R_F values: $PGF_{2\alpha}$ 0.17, 6-oxo-$PGF_{1\alpha}$ 0.12; elution with 2 ml of methanol + 2% acetic acid → filtration → evaporation → redissolution in citric acid—phosphate buffer, pH 3.8 → extraction two times with ether → evaporation → + 100 µl of 10% methoxyamine · HCl in pyridine → incubation overnight at room temperature → evaporation → methylation in 100 µl of methanol + 500 µl of ethereal diazomethane [3], 5 min at room temperature → evaporation → redissolution in dichloromethane → chromatography through a column (I.D. 5 mm) filled with 1 g of Sephadex LH-20 in dichloromethane → elution with 2 ml of dichloromethane → evaporation → addition of 20 µl of a mixture of hexamethyldisilazane—trimethylchlorosilane—pyridine (3:1:9) → splitless injection of 5 µl.

Instrumental conditions

A Finnigan 9610 gas chromatograph coupled to a Finnigan 4000 mass spectrometer with a positive ion electron-impact (EI) PCI device and an Incos data system are used. The gas chromatograph is equipped with a fused silica column coated with the chemically bonded DB-1 (30 m × 0.25 mm I.D.). The injector and the transfer line are kept at 260°C. The column is kept at 100°C for 1 min after injection, then heated with an increase of 40°C/min to 200°C and then at 3°/min to 300°C. The retention time of $PGF_{2\alpha}$ is 24 min, that of 6-oxo-$PGF_{1\alpha}$ 27 min. A shorter program resulted in inferior accuracy. The MS conditions are: CI with ammonia, ion source at 280°C, electron energy 120 eV, emission current 0.1 A.

RESULTS

The most prominent fragments in the higher mass range were monitored;

they are 405 for $PGF_{2\alpha}$ and 409 for its tetradeuterated form. For 6-oxo-$PGF_{1\alpha}$ we measured the mass 540 and 544 for the tetradeuterated $PGF_{1\alpha}$ (Fig. 1). Calibration curves were established for 30—2400 pg of each PG injected together with 10 ng of each in the tetradeuterated form. For 6-oxo-$PGF_{1\alpha}$ a correlation coefficient of 0.9989, an intercept of 0.004 and a slope of 0.021 were found. The correlation coefficient for $PGF_{2\alpha}$ was 0.9987, the slope 0.038 and the intercept 0.005. The standard deviation calculated from five parallel assays of pure PGs is about 2.5% and lies within 5% for urine samples. Since fragmentation in the ion source is slightly different for natural and for

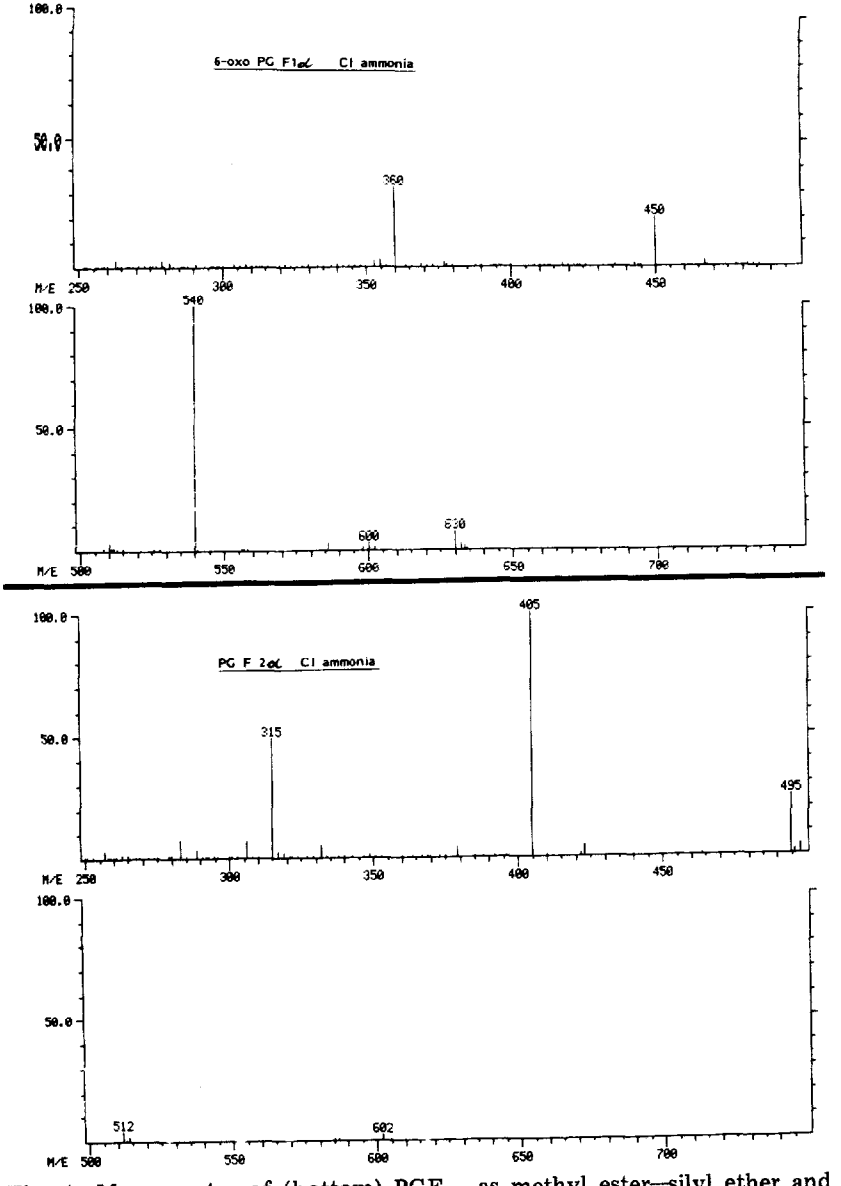

Fig. 1. Mass spectra of (bottom) $PGF_{2\alpha}$ as methyl ester—silyl ether and (top) of 6-oxo-$PGF_{1\alpha}$ as methyl ester—methyloxime—silyl ether obtained by chemical ionisation with ammonia as CI gas.

tetradeuterated PGs and because of the influences of the different source parameters on the fragmentation pattern, we establish a new calibration curve every day. The variations observed from day to day lie within 10%.

The detection limit for urine samples is 20 pg of each PG per ml urine (Fig. 2). For urine we detected 25—700 pg of 6-oxo-$PGF_{1\alpha}$ per mg creatinine. The values for $PGF_{2\alpha}$ are about ten times higher. The number of urines so far investigated, however, is too small to give reference values.

The specificity of the method is given by liquid—solid extraction, different chromatographic steps, formation of derivatives, GC on a 30-m fused silica column and selected ion monitoring (SIM) by MS.

The accuracy is ensured by addition of both PGs in tetradeuterated form as tracer and as internal standard. The overall recovery lies at 50% for urine samples. The losses, however, are compensated for by calculation via the internal standard.

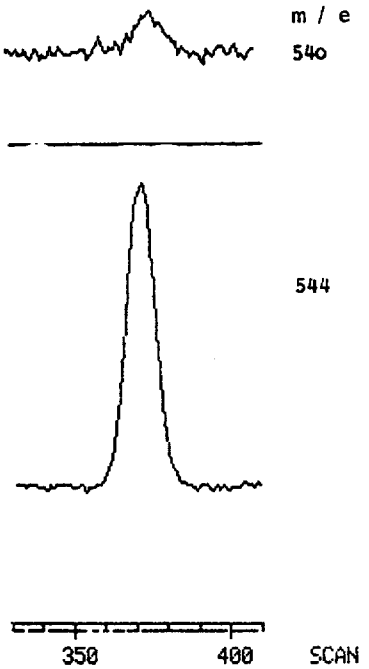

Fig. 2. Ion chromatogram obtained from urine showing the lower detection limit. The peak obtained for the mass 544 corresponds to 50 ng of tetradeuterated 6-oxo-$PGF_{1\alpha}$ added to 20 ml of urine. The amount of urinary 6-oxo-$PGF_{1\alpha}$ was calculated from the peak obtained for the mass 540 to be approximately 20 pg/ml urine.

DISCUSSION

The high purification of urine extracts combined with the addition of tetradeuterated PGs in great excess as tracers and as internal standards makes it possible to measure with PCI amounts as low as 20 pg PG per ml urine with high specificity and accuracy. A different behaviour of the tetradeuterated and

the natural PGs was not observed during the entire procedure, except during GC where the tetradeuterated PGs have slightly shorter retention times (the difference between labelled and unlabelled PG is about 4 sec at a retention time of 24 and 27 min). Further, we observed different fragmentation in the ion source in that the peak areas of identical amounts of labelled and unlabelled PG are not identical. These differences, however, are compensated for by the calibration curve. The results obtained for urinary $PGF_{2\alpha}$ correspond to those published by Seyberth et al. [4]. Those obtained for 6-oxo-$PGF_{1\alpha}$ can not be compared since we found only two methodological descriptions [2, 5] but no data.

Compared with the method for determinations from the serum [2] we use higher amounts for washing the columns to eliminate impurities from the urine. Further, we make the TLC purification step prior to derivatisation as the free PGs are better separated from interfering substances from the urine or from cholesterol if cell incubations are investigated. The difficulties reported [2] for methyloxime formation after TLC were overcome by our extraction procedure from the silica gel. Finally, methyl esters were used instead of pentafluorobenzyl esters since when working with PCI–MS these substances gave better results.

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