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Note

Determination of 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2 in urine by high-performance liquid chromatography and radioimmunoassay

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6-Keto-prostaglandin $F_{1\alpha}$ (6ketoPGF$_{1\alpha}$) and thromboxane B_2 (TXB_2) are the non-enzymatic hydrolysis products of prostacyclin (PGI_2) and thromboxane A_2 respectively [1]. Both these compounds have been found in urine [2,3] and their excretion rate may reflect renal synthesis [3,4]. The parent compounds are formed both in the kidney and elsewhere in the body [1] and hydrolyze rapidly to 6ketoPGF$_{1\alpha}$ and TXB_2. If formed in extra-renal sites they then can be metabolised to several other compounds. When PGI_2, 6ketoPGF$_{1\alpha}$ or TXB_2 are injected intravenously, little of each appears in urine but several metabolites of each are excreted [5,6]. These metabolites and other substances could conceivably interfere with the determination of 6ketoPGF$_{1\alpha}$ and TXB_2 by radioimmunoassay. By subjecting urine to complex extraction and purification procedures it is hoped that potentially interfering substances will be separated from the compounds of interest, but this hypothesis is difficult to test because of the lack of availability of all possible metabolites. Because reversed-phase high-performance liquid chromatography (HPLC) is a powerful separation technique for lipids we applied it to validate our extraction, purification and radioimmunoassay procedure.

EXPERIMENTAL

Materials and solvents

Pure standards of 6ketoPGF$_{1\alpha}$ and TXB_2 were donated by the Upjohn Company (Kalamazoo, MI, U.S.A.). [^{3}H]6ketoPGF$_{1\alpha}$ (150 Ci/m*M*) and [^{3}H]TXB_2 (100 Ci/m*M*) were purchased from New England Nuclear (Montreal, Canada) and used without further purification. All glass columns for Sephadex LH-20 were purchased from Johns Scientific (Toronto, Canada). Liquid scintil-

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lation counting was performed on a Nuclear Chicago Mark II instrument using 9 ml Aquasol (New England Nuclear). All other solvents were of the highest available purity.

Extraction of 6ketoPGF$_{1\alpha}$ from urine

Urine (10 ml) to which a tracer amount (1000 cpm) of [^{3}H]6ketoPGF$_{1\alpha}$ had been added was brought to pH 3.0 with 1.0 *M* hydrochloric acid and applied to a 6-ml prepacked, disposable octadecyl (C_{18}) column (J.T. Baker, Phillipsburgh, NJ, U.S.A.). The column was successively eluted with 20 ml of 15% methanol, 20 ml hexane and 15 ml methyl formate. The methyl formate fraction, containing the bulk of the 6ketoPGF$_{1\alpha}$, was evaporated to dryness under nitrogen, the residue taken up in 1 ml of a solvent system composed of chloroform—heptane—methanol—acetic acid (100:100:10:2) and applied to a 130 × 10 mm glass Sephadex LH-20 column. This column had previously been equilibrated with chloroform—heptane—methanol—acetic acid (100:100:30:2). After the sample was applied, the column was successively eluted with 10 ml of the original solvent, 9 ml of chloroform—heptane—methanol—acetic acid (100:100:20:2), and 20 ml of chloroform—heptane—methanol—acetic acid (100:100:25:2). In this latter fraction 78±3% (S.E.M.) (n=13) of the added [^{3}H]6ketoPGF$_{1\alpha}$ was contained. The sample was evaporated to dryness under nitrogen in a 40-ml conical centrifuge tube and stored for up to three days at −20°C before assay.

Extraction of TXB$_2$ from urine

Ten ml of urine, to which 1000 cpm of [^{3}H]TXB$_2$ had been added was titrated to pH 7.0 with 1.0 *M* potassium hydroxide and washed with 15 ml hexane to remove neutral lipids. The aqueous layer was brought to pH 3.5 with concentrated formic acid and extracted with 30 ml chloroform. The extract was evaporated under nitrogen, taken up in 0.4 ml of chloroform—heptane—ethanol—acetic acid (100:100:10:2) then applied to a 130 × 10 mm Sephadex LH-20 column. The column was eluted with the following solvent systems in sequence (chloroform—heptane—ethanol—acetic acid) 10 ml (100:100:10:2), 12 ml (100:100:20:2) and 20 ml (100:100:25:2). Recovery of added [^{3}H]TXB$_2$ was 64±3% (S.E.M.) (n=13). The final fraction, containing the TXB$_2$ was evaporated under nitrogen in a 40-ml conical centrifuge tube and stored at −20°C for up to three days prior to assay.

Radioimmunoassay procedures

Radioimmunoassays were performed in 75 × 10 mm polystyrene tubes. All reagents were diluted in Tris–PVP buffer (6.08 g Tris base, 9.0 g sodium chloride, 1.0 g polyvinylpyrrolidone (MW = 40,000), 3.4 ml of 0.5 *M* magnesium sulfate and 1.6 ml of 0.1 *M* calcium chloride, 2.0 ml of 10 *M* hydrochloric acid, per l water, pH 7.4). All assays were performed in duplicate. For 6ketoPGF$_{1\alpha}$ 5000 cpm [^{3}H]6ketoPGF$_{1\alpha}$ were added to tubes containing either buffer alone, known amounts of 6ketoPGF$_{1\alpha}$ (5—250 pg) or aliquots of urine extracts. Rabbit antiserum to 6ketoPGF$_{1\alpha}$ (supplied by Dr. P.V. Halushka, Medical University of South Carolina, Charleston, SC, U.S.A.) was added in a final dilution (assay volume 450 μl) of 1:25,600. The tubes were incubated

12—18 h at 4°C, and separation of bound and free [^{3}H]6ketoPGF$_{1\alpha}$ was achieved with 1.0 ml dextran—charcoal (1:10, w/w). Standard curves were constructed using Scatchard plots from which unknowns were calculated after subtracting the mass of [^{3}H]6ketoPGF$_{1\alpha}$.

A similar procedure was used for radioimmunoassay of TXB_2. To tubes containing either buffer, known amounts of TXB_2 (10—750 pg) or aliquots of urine extracts were added 5000 cpm [^{3}H]TXB_2. Rabbit antiserum to TXB_2 (again supplied by Dr. Halushka) was added to each tube in a final dilution of 1:20,000 for the total assay volume of 200 μl. Tubes were incubated at 37°C for 3 h. Separation of bound and free [^{3}H]TXB_2, using dextran—charcoal, and analysis were identical to the 6ketoPGF$_{1\alpha}$ radioimmunoassay.

HPLC experiments

Known amounts of 6ketoPGF$_{1\alpha}$ or TXB_2 were added to 10-ml aliquots of urine from a normal volunteer and extracted as detailed above. For these extractions, recovery calculation was based only on the amount assayed by HPLC. In some experiments, the amount of tritiated standard was increased 10—20 fold to allow measurement of radioactivity in fractions collected after HPLC.

The HPLC hardware consisted of a M-6000 pump, a U6K universal injector, a Model 480 UV detector, a Model 720 system controller and a Model 730 data module (Waters Assoc., Milford, MA, U.S.A.). Fractions were collected after the solvent had passed through the detector. A Waters Fatty Acid Analysis steel jacketed column (300 × 3.9 mm, particle size 10 μm) was used. Prostanoids were isocratically eluted at 1.5 ml/min with a mobile phase consisting of 2 m*M* potassium dihydrogen phosphate—acetonitrile (70:30), pH 3.5. The detector was adjusted to 194 nm with mobile phase in the reference cell. Fractions (0.75 ml) were collected after HPLC and subjected to radioimmunoassay as above. Where larger amounts of tritiated standards were added to the original urine specimens, the fractions were counted for tritium.

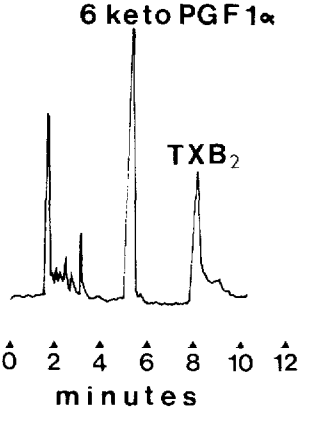

Fig. 1. Chromatogram of 6ketoPGF$_{1\alpha}$ and TXB_2 (100 ng each) in mobile phase. Retention times: 6ketoPGF$_{1\alpha}$ = 5.05 min; TXB_2 = 7.90 min.

RESULTS

Standard curves

Fig. 1 is a HPLC chromatogram of 6ketoPGF$_{1\alpha}$ and TXB_2, 100 ng of each injected in mobile phase. Retention times are 5.05 and 7.90 min, respectively. Standard curves, were linear over the range 25—100 ng. Regression lines relating area of the peak in mm^2 (Y) to ng of prostanoid added (X) were: 6ketoPGF$_{1\alpha}$, $Y=-3.5+5.29X$ ($r=0.998$); TXB_2, $Y=3.3+3.2X$ ($r=0.997$).

Radioassay and radioimmunoassay of HPLC fractions

Figs. 2 and 3 show chromatograms of urine extracts. The upper and lower

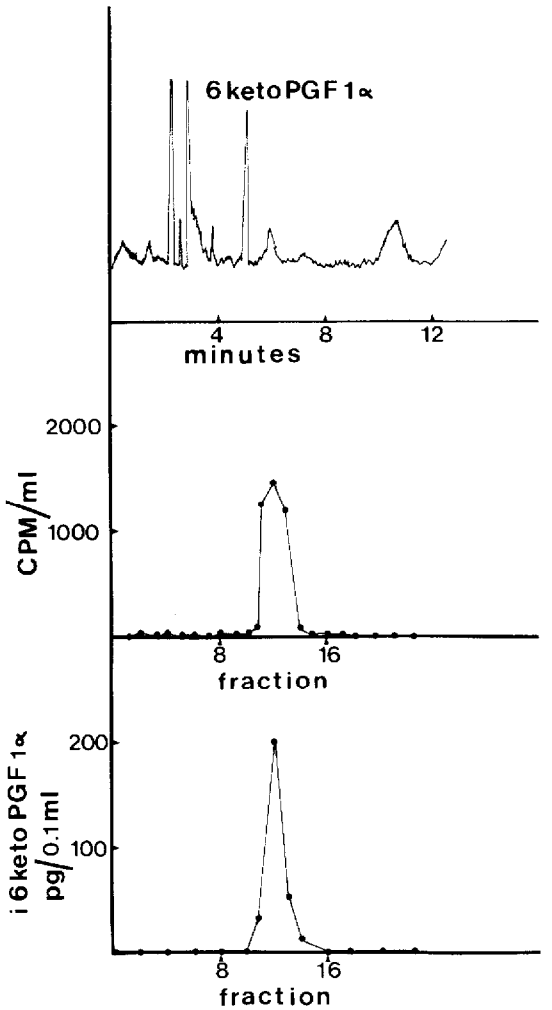

Fig. 2. Chromatograms of urine extracts; 100 μg authentic 6ketoPGF$_{1\alpha}$ or 10,000 cpm [^{3}H]6ketoPGF$_{1\alpha}$ added to 10 ml urine. Upper panel: HPLC chromatogram showing peaks representing more polar materials with early retention times. Middle panel: radioactivity per fraction. Lower panel: immunoreactive (i) 6ketoPGF$_{1\alpha}$ per fraction. The early peaks seen in the upper panel do not contain radioactivity or immunoreactive 6ketoPGF$_{1\alpha}$. The peaks in the lower two panels are delayed 1—2 min due to the volume from the UV detector to the fraction collector.

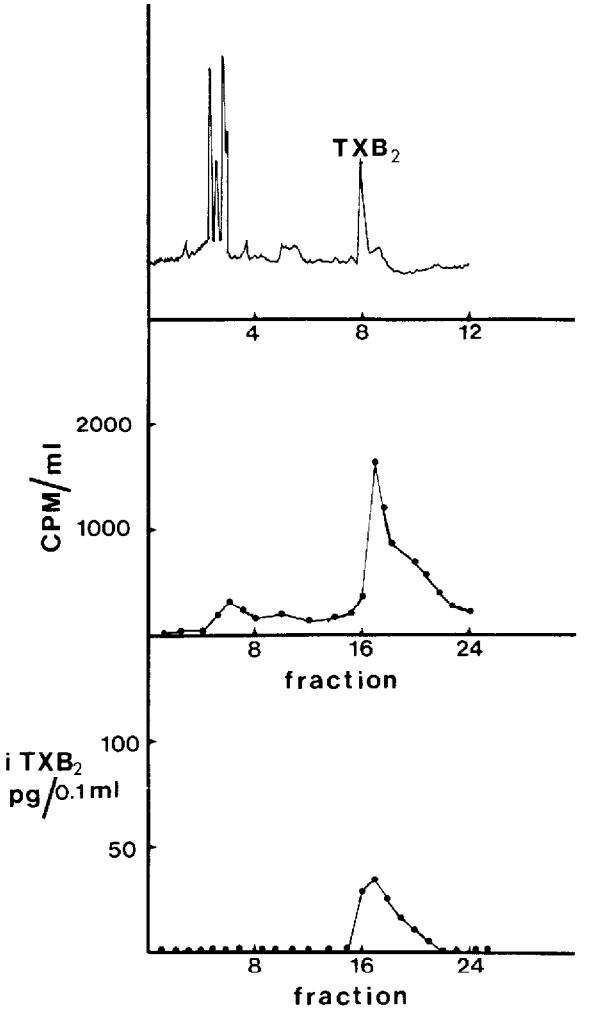

Fig. 3. Chromatograms of urine extracts; 50 μg authentic TXB_2 or 10,000 cpm [^{3}H]TXB_2 added to 10 ml urine. Upper panel HPLC chromatogram. Middle panel: radioactivity per fraction. Lower panel: immunoreactive (i) TXB_2 per fraction.

panels of each figure represent a 10-ml urine specimen to which 100 μg of each cold standard was added before extraction and purification. The middle panels represent similar urine aliquots to which tritiated compounds were added. It can be seen that the only HPLC peaks containing significant tritium or immunoreactive 6ketoPGF$_{1\alpha}$ and TXB_2 correspond to those with the retention times of authentic compounds. We take this result to mean that our extraction and purification procedures removed the bulk of potentially immunoreactive interfering substances.

The peaks drawn for tritium quantitation and for immunoassayable substance are somewhat more broad than those representing the HPLC chromatograms. This is probably due to mixing in the tubing and the fraction collector of material after its passage through the UV detector.

Recovery of added compounds

Table I shows the recovery of added 6ketoPGF$_{1\alpha}$ and TXB_2 to urine samples by HPLC. The values in the table represent the absolute mass of each prostanoid recovered at each concentration of prostanoid added. Three urine aliquots were measured at each concentration and the results averaged. It can be seen that the recovery is linear and averages 82% for 6ketoPGF$_{1\alpha}$ and 69% for TXB_2. These values are very similar to the recoveries obtained when tritiated standards were extracted from urine (78±3% and 64±3%, respectively). No HPLC peaks corresponding to either compound were seen when normal urine was extracted and assayed with the a.u.f.s. setting at 0.02. Given the recoveries (80 and 70%) and the minimal detectable level (25 ng) measurable by HPLC, we conclude that normal urine contains less than 4 ng/ml of each.

TABLE I

RECOVERY OF AUTHENTIC STANDARDS ADDED TO URINE BY HPLC

	Added (μg/10 ml)				
	0	25	50	100	200
	Recovery (μg/10 ml)				
6ketoPGF$_{1\alpha}$	0	20.3	39.5	85	not done
TXB_2	0	not done	38.5	65	130

Normal values

Thirteen normal volunteers (9 males, 4 females) aged 19–47 years who had received a 20 ml/kg oral water load excreted 551±63 ml (S.E.M.) of urine in a 4-h period. They excreted 0.11±0.03 (S.E.M.) ng/ml TXB_2 and 0.27±0.06 ng/ml 6ketoPGF$_{1\alpha}$. There was no correlation between the excretion rate of either compound and urine volume: for volume vs. 6ketoPGF$_{1\alpha}$ excretion rate, $r=-0.032$, for volume vs. TXB_2 excretion rate $r=0.379$.

DISCUSSION

The determination of prostanoids in biological specimens by radioimmunoassay suffers from uncertainty due to undetermined effects of potentially cross-reacting substances. While antisera used for radioimmunoassays (including our own; [7,8]) are commonly tested for cross-reactivity with other prostanoids and metabolites, the concentration of these compounds in biologic samples is usually unknown. Thus, if a compound is in 1000-fold excess, a cross-reactivity of 0.1% becomes very significant. Extraction and purification techniques are used to reduce the concentration of interfering substances but they should be validated. By subjecting our extracts to HPLC we showed a number of extra peaks which could represent potentially interfering substances. However, these peaks did not contain cross-reacting material when tested by radioimmunoassay. Furthermore, when urine was enriched with a large amount of tritiated 6ketoPGF$_{1\alpha}$ or TXB_2 and subjected to extraction, purification and HPLC, tritium appeared only in the fractions corresponding to authentic com-

pounds. While these results do not constitute absolute proof of the molecular specificity of our extraction and radioimmunoassay procedures it seems very unlikely that potentially interfering substances would have identical retention times on both the Sephadex LH-20 column and the HPLC column plus be immunoreactive. We are therefore, lead to the conclusion that our assay procedures are specific for urine 6ketoPGF$_{1\alpha}$ and TXB$_2$.

The HPLC procedure described in this paper can be used to measure reproducably as little as 25 ng of either 6ketoPGF$_{1\alpha}$ or TXB$_2$. This equals the reported sensitivity of other reported HPLC methods [9,10]. Such sensitivity results from performing the assay at a UV wavelength of 194 nm, which is near the maximal absorbance for most prostanoids [9]. Previously-described mobile phases cannot be used satisfactorily at such low UV wavelengths [10]. Secondly, we have found that these compounds resolve optimally when the pH of the mobile phase is maintained at 3.5.

Desiderio et al. [11] have reported the use of a volatile triethylamine—formic acid buffer system for wavelengths below 200 nm. Others have used a solvent system comprising of phosphoric acid and acetonitrile [7]. However, we have found that substantial baseline noise occurs with the triethylamine solvent system while the combination of phosphoric acid and acetonitrile was found to buffer inadequately at pH 3.5. Our solvent system comprised of 2 m*M* potassium dihydrogen phosphate—acetonitrile (70:30) offers excellent UV transparency at 194 nm, gives good baseline stability and buffers well at pH 3.5. However, even with the improvement in sensitivity, normal levels of 6ketoPGF$_{1\alpha}$ and TXB$_2$ in urine are not easily measured by HPLC assay but can be conveniently detected by radioimmunoassay.

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REFERENCES

1 S. Moncada and J.P. Vane, Pharmacol. Rev., 30 (1979) 293.
2 G. Ciabattoni, F. Pugliese, G.A. Cinotti, G. Stirati, R. Ronci, G. Castrucci, A. Pierucci and C. Patrono, Eur. J. Pharmacol., 60 (1979) 181.
3 T.W. Wilson, C.B. Loadholt, P.J. Privitera and P.V. Halushka, Hypertension, 4 (1982) 634.
4 A. Hornych, M. Safar, J. Bariety, A. Somon, G. London and J. Levenson, Prostaglandins Leukotrienes and Med., 10 (1983) 145.
5 L.J. Roberts, B.J. Sweetman and J.A. Oates, J. Biol. Chem., 253 (1980) 5305.
6 B. Rosenkranz, C. Fisher, K.E. Weimer and J.C. Frolich, J. Biol. Chem., 255 (1980) 10194.
7 W.C. Wise, J.A. Cook, T. Eller and P.V. Halushka, J. Pharmacol. Exp. Ther., 215 (1980) 160.
8 R.M. Burch, D.R. Knapp and P.V. Halushka, J. Pharmacol. Exp. Ther., 210 (1979) 344.
9 A. Teragno, R. Rydzik and N.A. Terragno, Prostaglandins, 21 (1981) 101.
10 T. Eling, R. Warnock, D. Dick and B. Tainer, Prostaglandins and Med., 5 (1980) 345.
11 D.M. Desiderio, M.D. Cunningham and J.A. Trimble, J. Liquid Chromatogr., 4 (1981) 1261.