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Simplified assay for the quantification of 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$ by gas chromatography–mass spectrometry

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Abstract

Endogenous prostacyclin production is best assessed by the measurement of its excreted metabolites, of which a major one is 2,3-dinor-6-ketoprostaglandin $F_{1\alpha}$ (2,3-dinor-6-keto-$PGF_{1\alpha}$). Gas chromatographic–mass spectrometric (GC–MS) assays have been developed for this compound but are cumbersome and time-consuming. We now report a modified assay for the measurement of 2,3-dinor-6-keto-$PGF_{1\alpha}$ employing GC–MS in which sample preparation time is markedly shortened by replacing a number of extraction steps with reversed-phase column extraction and by modifying derivatization procedures. Precision of the assay is ± 5% and the accuracy is 98%. The lower limit of detection in urine is approximately 15 pg/mg creatinine. Normal urinary levels of this metabolite were found to be 141 ± 54 pg/mg creatinine (mean ± S.D.). Urinary excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ is markedly altered in situations associated with abnormalities of prostacyclin generation when quantified using this assay. Thus, this assay provides a sensitive and accurate method to assess endogenous prostacyclin production and to further explore the role of this compound in human health and disease.

1. Introduction

Prostacyclin (PGI_2) is a major cyclooxygenase product of the vascular endothelium [1,2]. It is produced in increased amounts in disorders associated with endothelial damage such as atherosclerosis and myocardial infarction and it is believed that PGI_2 may be an important regulator of vascular tone [3,4]. Prostacyclin is an unstable compound which quickly undergoes degradation to its biologically inactive hydrolysis product, 6-keto-prostaglandin (PG)$F_{1\alpha}$ [5]. Although quantification of 6-keto-$PGF_{1\alpha}$ in urine has been used to assess endogenous prostacyclin production, it is recognized that a significant proportion of this compound may derive from local production in the kidney, and thus measurement of urinary 6-keto-$PGF_{1\alpha}$ levels may not be a useful indicator of systemic prostacyclin production [6]. On the other hand, the urinary excretion of a major metabolite of PGI_2, 2,3-dinor-6-keto-$PGF_{1\alpha}$, has been shown to accurately reflect systemic prostacyclin production [7,8].

Several assays have been developed to measure 2,3-dinor-6-keto-$PGF_{1\alpha}$ [9–11]. They rely on either immunoassay techniques or gas chromatography (GC)–mass spectrometry (MS). Im-

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munoassays of eicosanoids are generally facile and applicable to large sample numbers but suffer from a lack of specificity [12]. MS assays, on the other hand, while often cumbersome, are more specific for the measurement of prostaglandins [12]. One of the more commonly used GC–MS assays for the quantification of 2,3-dinor-6-keto-$PGF_{1\alpha}$ was originally described by Falardeau *et al.* [9]. It employs a series of differential extractions of biological fluids based on the fact that the molecule reversibly lactonizes under acidic conditions, followed by derivatization, purification by thin-layer chromatography (TLC) and analysis by MS. While this method often gives accurate results, it is extremely cumbersome and time consuming, generally requiring 2 full working days to quantify 8–10 urine samples. In an effort to overcome this problem, we report modifications of the assay in which many of the differential extraction steps are replaced by reversed-phase column extraction. In addition, derivatization procedures are modified. These changes make the analysis of 2,3-dinor-6-keto-$PGF_{1\alpha}$ far simpler and less time consuming.

2. Experimental

2.1. Clinical studies and sample collection

To determine normal urinary levels of 2,3-dinor-6-keto-$PGF_{1\alpha}$, 24-h urine collections were obtained from healthy volunteers ($n = 10$) taking no medications. They were processed immediately. Two studies were performed to evaluate the ability of the assay described below to detect variations in endogenous prostacyclin production after pharmacological intervention *in vivo*. In the first study, 3 volunteers were administered bradykinin intravenously at dosages ranging from 0.5 to 25 μg/kg/min until symptoms consisting of vasodilation or hypotension occurred. Bradykinin is known to induce the endogenous release of large quantities of PGI_2 in association with these symptoms [13]. Twenty four hour pre-infusion and 1-h post infusion urine collections were obtained from each volunteer and analyzed for 2,3-dinor-6-keto-$PGF_{1\alpha}$. In a second study, the same 3 volunteers were administered indomethacin 50 mg orally three times a day for 4 days and a 24-h urine collection beginning on the fourth day was obtained and processed for 2,3-dinor-6-keto-$PGF_{1\alpha}$. Informed consent was obtained prior to all clinical studies.

2.2. Chemicals and reagents

Methoxyamine hydrochloride, pentafluorobenzyl bromide, and N,N-diisopropylethylamine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethylformamide and undecane were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Supelco (Bellefonte, PA, USA). Octadecyl (C_{18}) Sep-Pak cartidges were purchased from Waters Associates (Milford, MA, USA). TLC plates (silica gel 60ALK6D) were obtained from Whatman International (Maidstone, UK). All organic solvents were purchased from Baxter Healthcare (Burdick and Jackson Brand, McGaw Park, IL, USA). 6-Keto-$PGF_{1\alpha}$ was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). 2,3-Dinor-6-keto-$PGF_{1\alpha}$ and [$^2H_4$]2,3-dinor-6-keto-$PGF_{1\alpha}$ were synthesized and kindly provided by Dr. Alan Brash, Vanderbilt University.

2.3. General extraction and purification procedure

The method outlined in Fig. 1 for the quantification of 2,3-dinor-6-keto-$PGF_{1\alpha}$ differs significantly from that originally described by Falardeau *et al.* [9]. The differences between the two assays are discussed in the Results and discussion section.

To 3 ml of urine is added 1 ng [$^2H_4$]2,3-dinor-6-keto-$PGF_{1\alpha}$ and the sample is vortex-mixed well. It is then acidified to pH 3 with 1 mol/l HCl and allowed to stand at room temperature for 5 min. Under these conditions, 2,3-dinor-6-keto-$PGF_{1\alpha}$ exists essentially entirely as a lactone. The sample is then applied to a C_{18} Sep-Pak preconditioned with 5 ml methanol followed

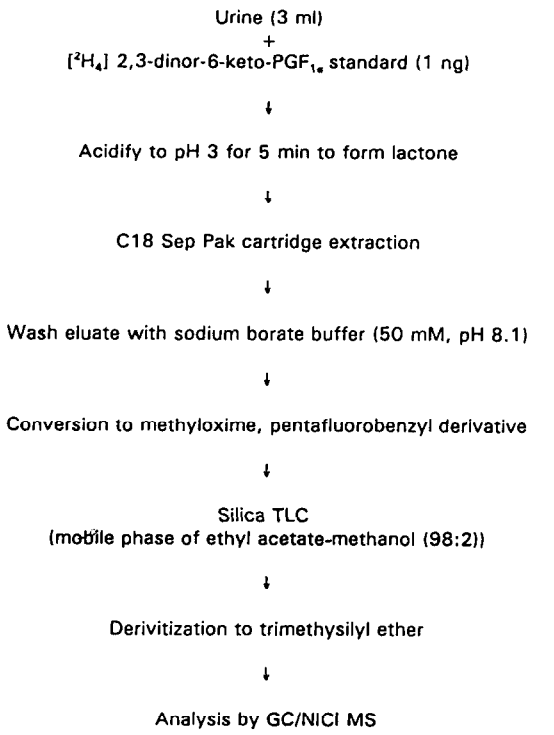

Fig. 1. Summary of method employed for the quantification of 2,3-dinor-6-keto-PGF$_{1\alpha}$.

by 5 ml H_2O (pH 3). The sample is rinsed with 10 ml H_2O (pH 3) followed by 10 ml heptane. The sample is then eluted with 10 ml ethyl acetate–heptane (50:50, v/v) into a scintillation vial. The eluate is dried over 10 g of anhydrous sodium sulfate, transferred to a reactivial, and evaporated under nitrogen. The residue is reconstituted in 1 ml of dichloromethane and washed twice with 2 ml of a 50 mmol/l sodium borate buffer (pH 8.1), discarding the aqueous layer after each wash. At this step, 2,3-dinor-6-keto-PGF$_{1\alpha}$ still remains in the lactone form and does not extract into the aqueous phase. Non-lactonized fatty acids that are present as carboxylate anions, however, are readily extracted into the buffer layer.

Subsequently, the organic phase is dried under nitrogen and reconstituted in 50 μl of acetonitrile. To this is added 200 μl of a 3% solution of methoxyamine hydrochloride into water and the mixture is incubated for 45 min at room temperature. This step results in opening of the lactone ring of the molecule and simultaneous derivatization of the keto group. Simultaneous opening and derivatization by methoxyamine hydrochloride has been shown to occur for other lactone rings such as the major urinary metabolite of PGD_2 [14]. The sample is then extracted with 1 ml of dichloromethane and dried under nitrogen. The residue is derivatized to the pentafluorobenzyl (PFB) ester by the addition of 40 μl PFB bromide (10% by volume in acetonitrile) and 20 μl N,N-diisopropylethylamine (10% by volume in acetonitrile) and incubating for 20 min at 37°C. The sample is then dried under nitrogen and reconstituted in 50 μl of methanol. It is applied to a silica TLC plate and developed to the top in a solvent system of ethyl acetate–methanol (98:2, v/v). The methyloxime, PFB ester derivative of 6-keto-PGF$_{1\alpha}$ (5 μg) is chromatographed on a separate lane and visualized with 10% phosphomolybdic acid after heating. The 6-keto-PGF$_{1\alpha}$ derivative is used as a TLC standard rather than derivatized 2,3-dinor-6-keto-PGF$_{1\alpha}$ to avoid any contamination of the biological samples. Using this solvent system, the 2 methyloxime isomers of derivatized 6-keto-PGF$_{1\alpha}$ are visualized with an $R_F = 0.25$ for the upper band and $R_F = 0.21$ for the lower band. Derivatized 2,3-dinor-6-keto-PGF$_{1\alpha}$ migrates in a nearly identical manner with an $R_F = 0.25$ for the upper band and $R_F = 0.22$ for the lower band. The compounds in the sample migrating 1 cm on either side of the point midway between the two methyloxime isomers of derivatized 6-keto-PGF$_{1\alpha}$ are scraped from the silica and eluted with 1 ml of ethyl acetate. The sample is then dried under nitrogen and converted to a trimethylsilyl (TMS) ether derivative by addition of 20 μl BSTFA and 10 μl dimethylformamide. The samples are incubated at 37°C for 10 min and dried under nitrogen. The residue is redissolved for GC–MS analysis in 10 μl of undecane which has been stored over a bed of calcium hydride.

2.4. Gas chromatography–mass spectrometry

GC–negative-ion chemical ionization (NICI) MS is carried out using a Nermag R10-10C GC–

MS instrument interfaced with a DEC-PDP 11/23 plus computer system. GC is performed using a 15 m × 0.25 mm I.D., 0.25 μm film thickness, DB 1701 fused-silica capillary column (J and W Scientific, Folsom, CA, USA). The column temperature program runs from 190 to 300°C at 20°/min. Sample retention time is 6–7 min. Methane is used as the carrier gas at a flow-rate of 1 μl/min. Ion-source temperature is 250°C, electron energy 70 eV and filament current 0.25 mA. The major ion generated in the NICI mass spectra of the methyloxime, PFB ester, TMS ether derivative of 2,3-dinor-6-keto-$PGF_{1\alpha}$ is m/z 586 which represents the M − 181 (M − $CH_2C_6F_5$) carboxylate anion. For the deuterated standard, the corresponding ion is m/z 590. Quantification of endogenous 2,3-dinor-6-keto-$PGF_{1\alpha}$ in a urine sample is accomplished by selected-ion monitoring analysis of the ratio of intensities of m/z 586 to m/z 590.

3. Results and discussion

3.1. Assay modifications

The assay described herein differs in several important ways from that originally reported by Falardeau *et al.* [9]. These differences result in a marked shortening of the time required to process samples through the assay.

The first change concerns the equilibration time required for the initial cyclization of 2,3-dinor-6-keto-$PGF_{1\alpha}$ to the lactone. The previous method employed alkalinization of urine for 15 min to open any pre-existing lactone rings followed by acidification for up to 30 min to reform the lactone. We have found, however, that the recovery of a known amount of 2,3-dinor-6-keto-$PGF_{1\alpha}$ through the assay is the same whether or not urine is initially alkalinized. Further, the degree of cyclization of the molecule after 5 min is the same as after 15, 30 or 60 min (100% at all time points, $n = 4$). Thus, we believe that 5 min is adequate to ensure lactone ring formation of 2,3-dinor-6-keto-$PGF_{1\alpha}$ and that the initial alkalinization of the sample is unnecessary.

One of the more time-consuming aspects of the assay for urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ as described by Falardeau *et al.* is the need to perform an initial column extraction followed by no less than four separate extraction and back extraction steps coupled with a TLC procedure. We report, however, that all of these extraction steps are easily replaced by a C_{18} Sep-Pak column extraction followed by an aqueous wash of the organic eluate and subsequent TLC.

Thirdly, the assay has also been shortened by the observation that derivatization of 2,3-dinor-6-keto-$PGF_{1\alpha}$ with methoxyamine hydrochloride simultaneously opens the ring making prior opening as reported by Falardeau *et al.* unnecessary.

Finally, as originally described, 2,3-dinor-6-keto-$PGF_{1\alpha}$ was converted to the methyloxime derivative by the overnight incubation of samples in a solution of 3% methoxyamine hydrochloride in pyridine. We have found, however, that essentially complete formation (100 ± 6%) of the methyloxime derivative occurs after incubation of urine samples for 45 min in a solution of 3% methoxyamine in water. For this determination, complete cyclization was the amount of endogenous 2,3-dinor-6-keto-$PGF_{1\alpha}$ quantified by GC–MS in the same urine samples incubated in 3% methoxyamine in pyridine overnight. The importance of this modification is that a 2-day assay procedure is shortened to less than 1 day. It should be noted, however, that this shortened derivatization time is not novel since it has been noted that 2,3-dinor-6-keto-$PGF_{1\alpha}$ and other ketoprostaglandins can be methoximated in a period of time shorter than the 12–14 h originally reported by Falardeau [14–19].

3.2. Assay parameters and validation

A representative chromatographic tracing of 2,3-dinor-6-keto-$PGF_{1\alpha}$ obtained from the analysis of urine from a normal individual utilizing the assay outline herein is depicted in Fig. 2. As is evident, two methyloxime isomers are present for both the deuterated standard and endogenous compound. For quantification, the second methyloxime isomer in each chromatogram is compared. As is also apparent, the chromatographic peaks are entirely free of interfering impurities.

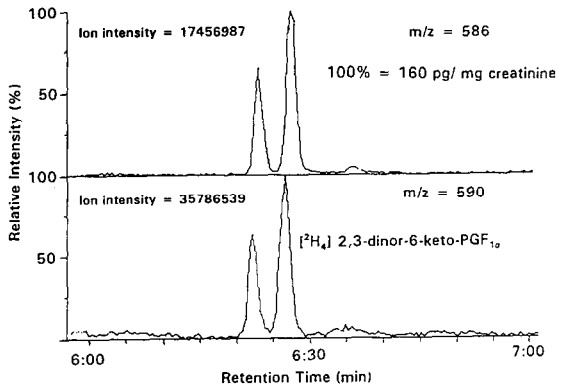

Fig. 2. Analysis of urine from a normal human volunteer for 2,3-dinor-6-keto-$PGF_{1\alpha}$. Endogenous 2,3-dinor-6-keto-$PGF_{1\alpha}$ is represented by the 2 peaks in the *m/z* 586 chromatogram and the deuterated standard by the 2 peaks in the *m/z* 590 chromatogram. The urinary level in this patient was 160 pg/mg creatinine.

Employing this assay, the lower limits of detection (signal-to-noise ratio of approximately 4:1) of 2,3-dinor-6-keto-$PGF_{1\alpha}$ in urine is in the range of 15 pg/mg creatinine using a deuteriated standard with a blank of 2–3 parts per thousand.

Several procedures were then performed to establish the accuracy and precision of this assay. Initially a standard curve was constructed by adding varying amounts of unlabelled 2,3-dinor-6-keto-$PGF_{1\alpha}$ to a fixed quantity of 1 ng of [$^2H_4$]2,3-dinor-6-keto-$PGF_{1\alpha}$ and the measured ratio of *m/z* 586 to *m/z* 590 to the expected ratio was compared. The assay was found to be linear over a 100-fold concentration range with the corresponding regression equation being (ratio *m/z* 586 to *m/z* 590) = 0.96 × (ratio amount unlabelled 2,3-dinor-6-keto-$PGF_{1\alpha}$ to deuterated 2,3-dinor-6-keto-$PGF_{1\alpha}$) − 0.01. The coefficient of variation was determined to be 0.999.

Precision was measured by analyzing six 3-ml aliquots of urine obtained from a normal volunteer. The mean of 3 replicate measurements of the ratio of *m/z* 586 to *m/z* 590 was determined for each sample. The precision was found to be ± 5%. Accuracy was assessed using the same urine. For this 310 pg of unlabeled 2,3-dinor-6-keto-$PGF_{1\alpha}$ were added to another four 3-ml aliquots of urine and reassayed. The amount of endogenous 2,3-dinor-6-keto-$PGF_{1\alpha}$ measured in the precision experiment was subtracted from the total and the accuracy of the assay to measure the added 2,3-dinor-6-keto-$PGF_{1\alpha}$ calculated. The accuracy was found to be 98%.

Recovery of 2,3-dinor-6-keto-$PGF_{1\alpha}$ employing this assay was in the range of 30–40%.

3.3. Urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ levels in normal humans

To establish the normal range of the urinary excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ using this assay, aliquots of urine from 24-h collections were obtained and analyzed from 10 healthy individuals. Normal levels were 141 ± 54 pg/mg creatinine (mean ± S.D.). These values are similar to levels reported by others [3,20].

3.4. 2,3-Dinor-6-keto-$PGF_{1\alpha}$ levels in clinical situations associated with alterations in prostacyclin production

We next examined the ability of this assay to assess alterations in endogenous prostacyclin production in humans. Others have reported that the intravenous administration of bradykinin to individuals induces marked flushing, hypotension and breathing difficulties associated with increases in endogenous prostacyclin production [13]. Thus, we examined whether infusions of bradykinin into healthy volunteers were associated with increases in the urinary excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ as quantified by the assay described herein. In these studies, three individuals were administered bradykinin intravenously at increasing doses ranging from 0.5 up to 25 μg/kg/min. Infusions were stopped when volunteers experienced clinical symptoms. Urine was assayed for 2,3-dinor-6-keto-$PGF_{1\alpha}$ before infusion and after the onset of clinical symptoms. The results are shown in Table 1. As is evident, in all three individuals, infusions of bradykinin markedly increased urinary excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ above baseline levels, with increases ranging from 3-fold to 5.4-fold.

Subsequently, each of the volunteers in the above study was administered the cyclooxygenase inhibitor indomethacin at a dose of 50 mg orally three times a day for 4 days. Non-steroidal anti-inflammatory agents have been shown to

Table 1
Effect of bradykinin infusion or indomethacin[a] on the excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ in 3 volunteers

Volunteer	2,3-Dinor-6-keto-$PGF_{1\alpha}$ excretion (pg/mg creatinine)		
	Baseline	+ Bradykinin	+ Indomethacin
1	61	326	24
2	100	330	26
3	183	557	28

[a] Details are given in the text under Clinical Studies and Sample Collection.

markedly decrease prostacyclin production *in vivo* [7,13]. The effects of this agent on urinary excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ are also shown in Table 1. As is evident, indomethacin markedly decreased the urinary excretion of this prostacyclin metabolite compared to baseline values, with decreases ranging between 60 and 85%.

4. Conclusions

In summary, we report a modified GC–MS assay for the measurement of 2,3-dinor-6-keto-$PGF_{1\alpha}$ based upon techniques originally reported by Falardeau *et al.* [9]. These modifications significantly shorten the time required to process and analyze samples. For example, no more than 8 to 10 samples can be processed and analyzed in a 2-day period using the method originally reported. However, employing the modified assay, approximately 15 samples can be processed and analyzed using this assay in a 6–7 h period. In addition, this assay is both sensitive and accurate and is able to detect alterations in prostacyclin production *in vivo*. Thus, this assay for 2,3-dinor-6-keto-$PGF_{1\alpha}$ should be a valuable tool to further study the role of prostacyclin in human health and disease.

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