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Short Communication

Simplification of the mass spectrometric assay for the major urinary metabolite of prostaglandin D_2

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ABSTRACT

The symptoms and hemodynamic alterations that accompany systemic mast cell activation have been attributed in large part to an excessive release of prostaglandin D_2 (PGD_2). Further, PGD_2 has been implicated in the adverse effects of some pharmacologic agents (*e.g.* nicotinic acid). Quantitation of the major urinary metabolite of PGD_2 has been invaluable in elucidating a role for PGD_2 in these clinical entities and in the biochemical diagnosis of the disease systemic mastocytosis. However, the stable-isotope mass spectrometric assay originally developed for quantification of this metabolite has been too cumbersome for routine use. We now report improvements in the assay that greatly increase its utility by shortening sample processing and eliminating the need for purification using thin-layer chromatography. The precision and accuracy of the modified assay was evaluated and found to be comparable with the previously described assay. These modifications potentially allow wider use of the assay to explore the role of PGD_2 in human disease and in the routine biochemical diagnosis of systemic mastocytosis and other disorders of mast cell activation.

INTRODUCTION

Many of the symptoms and the hemodynamic alterations that accompany episodes of systemic mast cell activation have been attributed in large part to excessive release of prostaglandin D_2 (PGD_2) from mast cells [1]. Further, PGD_2 has been implicated as the cause of the adverse effects of some pharmacologic agents (*e.g.* nicotinic acid-induced cutaneous vasodilation) [2]. Quantitation of the major urinary metabolite of PGD_2 (PGD-M), 9α,11β-dihydroxy-15-oxo-2,3,18,19-tetranorprost-5-ene-1,20-dioic acid, has been invaluable in elucidating a role for PGD_2 in these clinical entities [3]. A highly accurate and sensitive stable-isotope dilution gas chromatographic–negative-ion chemical ionization mass spectrometric (GC–NICI-MS) assay was developed for quantification of PGD-M but was labor-intensive and cumbersome [4]. Recently, we found that measurement of urinary levels of PGD-M is a more sensitive indicator of systemic disease in patients with mastocytosis than measurement of urinary histamine or the histamine metabolite, N^{τ}-methylhistamine (manuscript in preparation). This provided the incentive to evaluate whether the previously developed MS assay for PGD-M could be modified to increase efficiency, thus allowing its wider usage for both investigational

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TABLE I

COMPARISON OF THE ORIGINAL AND MODIFIED PGD-M ASSAY

Differences are highlighted with italics.

Step	Original assay	Modified assay
A	Add 0.88 ng internal standard to 1 ml urine and allow PGD-M to cyclize for 30 min	Add 0.88 ng internal standard to 1 ml urine and allow PGD-M to cyclize for 30 min
B	C_{18} Sep-Pak extraction	C_{18} Sep-Pak extraction
C	Silica Sep-Pak extraction	*Omitted*
D	Methylation	Methylation
E	Methoximation	Methoximation
F	Extraction from aqueous methoxyamine–HCl solution with ethyl acetate to second vial	*Addition of 1.5 ml borate buffer pH 9.1 to bring to neutral pH*
G	Addition of neutral borate buffer to evaporated extract and extraction of neutral lipids with ethyl acetate	*Extraction of neutral lipids with ethyl acetate (2 ml)*
H	Acidification of buffer to <pH 3 with 1 *M* HCl	Acidification of buffer to <pH 3 with 1 *M* HCl (~150 μl)
I	Extraction of PGD-M with ethyl acetate to third vial	*Extraction of PGD-M with dichloromethane (2 ml) and removal of aqueous top layer*
J	Formation of pentafluorobenzyl ester: 20 min reaction × 2	*Formation of pentafluorobenzyl ester: 20 min reaction × 1*
K	TLC	*Omitted*
L	Formation of trimethylsilyl ether derivative	*Formation of trimethylsilyl ether derivative directly*
M	Analysis by GC–NICI-MS–SIM of *m/z* 514 for endogenous compounds and *m/z* 522 for internal standard	Analysis by GC–NICI-MS–SIM of *m/z* 514 for endogenous compounds and *m/z* 522 for internal standard

purposes and in the routine biochemical diagnosis of disorders of mast cell activation.

In this report we describe modifications of the orginal MS assay for PGD-M which results in considerable simplification of the assay and reduces the time required to perform it without compromising the accuracy and sensitivity of PGD-M measurement.

EXPERIMENTAL

Unlabelled PGD-M was chemically synthesized as described by Prakash *et al.* [5] and then converted to the $^{18}O_4$-labeled derivative for use as an internal standard by the method of Murphy and Clay [6] by successive methylation and alkaline hydrolysis with $Li^{18}OH$. Dye mixture 1 for silica thin-layer chromatography (TLC) was obtained from Analtech (Newark, DE, USA). All other chemicals and reagents were obtained from sources described previously [4]. The modified method for the quantification of PGD-M is summarized in Table I and contrasted with the assay that was developed originally [4]. The differences between the two methods is discussed in detail in the Results section.

The GC and MS conditions were as described previously [4]. Briefly, analyses were performed with a Nermag R10-10C mass spectrometer (Fairfield, NJ, USA) operating in the negative-ion mode coupled to a Varian 6000 gas chromatograph (Sunnyvale, CA, USA) using a 15-m DB 1701-60N fused-silica capillary column (Supelco, Folsom, CA, USA). The GC oven was programmed 190 to 300°C at 20°C/min. Helium was used as the carrier gas at a flow-rate of 1 ml/min. Ion source temperature was 200°C, electron energy was 87 eV, and filament current was 0.25 mA.

RESULTS

PGD-M is analyzed as the O-methyloxime, monomethyl ester, mono(pentafluorobenzyl) es-

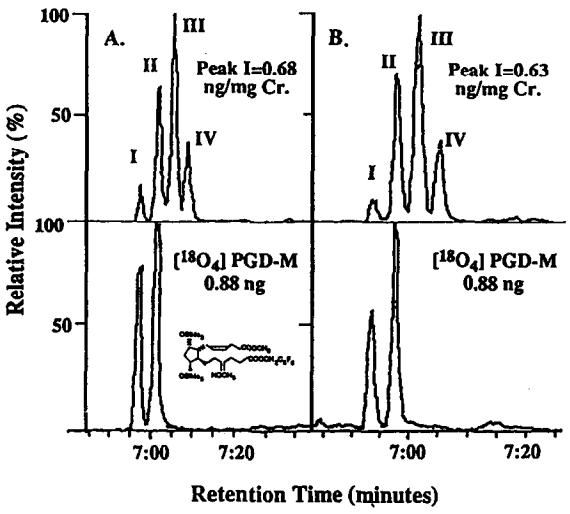

Fig. 1. Negative-ion chemical ionization selected ion current chromatograms obtained from analysis of the same urine for PGD-M using the originally developed assay (A) and the simplified assay (B). The top panels are the *m/z* 514 ion current chromatograms representing endogenous compounds and the bottom panels are the *m/z* 522 ion current chromatograms representing the *syn*- and *anti*-O-methyloxime isomers of the [$^{18}O_4$]PGD-M internal standard. The inset depicts the derivative of PGD-M formed. As discussed under Results, *m/z* 514 peak I represents an O-methyloxime isomer of endogenous PGD-M whereas most of peak II and all of peaks III and IV represent metabolites of non-cyclooxygenase-derived prostanoids (F_2-isoprostanes).

ter, bis(trimethylsilyl) ether derivative (Fig. 1, inset). A selected ion current chromatogram obtained from analysis of PGD-M in urine using the originally developed assay is shown in Fig. 1A. The major ion in the NICI mass spectrum of PGD-M is *m/z* 514 (M − 181, loss of $\cdot CH_2C_6F_5$). The analagous ion generated by the [$^{18}O_4$]PGD-M internal standard is *m/z* 522, and two peaks representing the *syn*- and *anti*-O-methyloxime isomers of [$^{18}O_4$]PGD-M are seen (Fig. 1A and B, bottom panels). However, in the *m/z* 514 ion current chromatograms (Fig. 1A and B, top panels), four peaks are present and have been designated by I–IV. Peak I can be suppressed approximately 90% by cyclooxygenase inhibitors and thus represents an O-methyloxime isomer of PGD-M [4]. On the other hand, peak II is only partially suppressed while the remaining peaks are unaffected by cyclooxygenase inhibitors and therefore, not derived from the activity of that enzyme. These peaks have been shown to represent metabolites of non-cyclooxygenase-derived PGF_2-like compounds (F_2-isoprostanes) [7]. Since essentially all of the material in peak I is produced by the cyclooxygenase enzyme and thus represents PGD-M exclusively, measurement of this peak has been used to precisely and accurately quantitate endogenous PGD-M [4].

Assay modification

The differences between the original and modified assays are outlined in Table I. The first important modification is the elimination of the silica solid-phase extraction step (step C). After extraction of the cyclized form of PGD-M from urine with a C_{18} Sep-Pak, the current assay proceeds directly to methylation of the upper sidechain followed by formation of the O-methyloxime isomers (steps D and E). Suprisingly, the silica solid-phase extraction was found not only to be unneccesary but detrimental. In the original assay, the differential extraction of PGD-M as a free acid from the bulk of methylated lipid was tedious requiring transfers to two additional reacti-vials (steps F–I). The second major modification involves simplification of this process by adding borate buffer pH 9.1 to the reacti-vials containing the acidic methoximation solution (75 μl of acetonitrile and 300 μl of 3% aqueous methoxyamine–HCl solution) to bring the pH to 7–8 (step F, modified assay). This allows ethyl acetate to be added to the same vial and the neutral lipids to be extracted from the PGD-M free acid which remains in the aqueous phase at neutral pH (step G, modified assay). This eliminates the need to extract lipids from the aqueous methoxyamine–HCl with ethyl acetate and transfer of the extract to another vial to be evaporated prior to resuspension in a neutral buffer. The assay modification then permits extraction of PGD-M into an organic phase in one reacti-vial simply by adjusting the pH to <3 with 1 *M* HCl and adding dichloromethane (step I, modified assay).

In addition to simplifying the extraction proce-

dure, performing the final extraction with dichloromethane instead of ethyl acetate proved to be very advantageous. The original ethyl acetate extractions carried some of the salt from the methoxyamine–HCl and borate buffer solutions into step J, formation of the pentafluorobenzyl ester derivative. Extraction with dichloromethane eliminates this residue which permits a single 20-min esterification reaction. Originally, we found it neccessary to repeat this step to assure adequate yield from the reaction. In addition, dichloromethane extraction makes possible the final major modification of the assay. For analysis of human urine, the trimethylsilyl ether derivative can now be formed directly after the formation of the pentafluorobenzyl ester rather than first undergoing purification by TLC (step K). All together, approximately 2 h of labor can be saved in addition to costly materials. Ten urine samples can routinely be prepared for analysis by MS in about 4 h.

Validation of precision and accuracy of the modified assay

Shown in Fig. 1 are the selected ion current chromatograms obtained from analysis of PGD-M in urine obtained from a normal volunteer. The similarity of the *m/z* 514 and *m/z* 522 selected ion current chromatogram obtained using the original assay method (Fig. 1A) and that obtained using the modified assay (Fig. 1B) is readily apparent. Further, the level of PGD-M measured by the modified assay (0.63 ng/mg creatinine) was essentially the same as the level measured by the original assay (0.68 ng/mg creatinine). The precision of the modifed assay was determined by measuring levels of PGD-M in five aliquots of the same urine and found to be ±8.5%. The precision reported for the original assay was reported as ±7.0% [4]. To ensure that the modified assay consistently eliminates any potential interfering impurities, levels of PGD-M measured in aliquots of 24-h urine collections from seven males and nine females were compared using the modified assay with and without incorporating the TLC purification step. No significant differences in levels measured by the two methods were observed (Table II). Furthermore, the modified assay was also found to be as accurate as the original assay for increased levels of PGD-M in urine. In a patient with documented systemic mastocytosis, the level of PGD-M in urine measured by the original assay was profoundly increased at 92 ng/mg creatinine (normal = 0.84 ± 0.36 ng/mg creatinine) and the level measured by the modified assay was essentially identical (95 ng/mg creatinine).

TABLE II

PGD-M VALUES DETERMINED USING THE MODIFIED PGD-M ASSAY INCORPORATING AND OMITTING THE TLC PURIFICATION STEP IN SIXTEEN HEALTHY INDIVIDUALS (SEVEN MALE AND NINE FEMALE)

Group	PGD-M (mean ± 1 S.D.) (ng/mg creatinine)	
	TLC	No TLC
All	0.84 ± 0.36	0.77 ± 0.30
Male	0.98 ± 0.42	0.88 ± 0.37
Female	0.73 ± 0.29	0.68 ± 0.19

DISCUSSION

Quantification of the major urinary metabolite of PGD_2 has proven to be a reliable approach to assess endogenous production of PGD_2 and explore its role in human disease, in particular disorders involving systemic mast cell activation [3]. However, the original MS assay method developed for measurement of PGD-M, although accurate, was cumbersome and inefficient making it unsuitable for routine diagnostic application. This provided the impetus to improve the efficiency of the assay. The modifications incorporated in the assay described herein retain the high degree of precision and accuracy of the original assay whilst shortening considerably the time required to perform the assay. Thus, the utility of this assay for PGD-M measurement, for both investigational purposes and routine clinical diagnosis of patients with systemic mastocytosis and

other disorders involving systemic mast cell activation, should be greatly enhanced.

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