*Journal of Chromatography*, 577 (1992) 1–7
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6302

# Gas chromatographic–mass spectrometric determination of 11-dehydrothromboxane $B_2$ in human urine

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(First received December 2nd, 1991; revised manuscript received January 27th, 1992)

ABSTRACT

The extension of a method for the determination of thromboxane $B_2$ ($TxB_2$), 2,3-dinor-$TxB_2$, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and 2,3-dinor-6-keto-$PGF_{1\alpha}$ to quantify 11-dehydro-$TxB_2$ in the same urinary sample is described. After phenylboronic acid and $C_{18}$ column chromatography, 11-dehydro-$TxB_2$, which is present in urine as the lactone and its corresponding hydroxy acid, was quantitatively converted into its lactone form for a thin-layer purification step and pentafluorobenzyl esterification. Quantification of eicosanoids was achieved by analysing their trimethylsilyl ethers with gas chromatography and negative-ion chemical ionization mass spectrometry. The overall recovery from urine for tritiated 11-dehydro-$TxB_2$ was 80%. The detection limit was 10 pg/ml. The method was applied to the determination of these eicosanoids in volunteers and in patients suffering from acute myocardial infarction.

## INTRODUCTION

11-Dehydrothromboxane $B_2$ (11-dehydro-$TxB_2$ is one of the major metabolites of $TxA_2$, a vasoconstrictor and stimulant of platelet aggregation with great importance in thrombosis, myocardial infarction or posttraumatic and postoperative consequences [1–3]. Because $TxA_2$ is not stable *in vivo*, the determination of its biologically stable metabolites, *e.g.* $TxB_2$, 2,3-dinor-$TxB_2$ and 11-dehydro-$TxB_2$, in blood and urine reflects the $TxA_2$ release.

Determination of $TxB_2$ in blood is necessarily associated with artifacts due to the invasive sampling technique [4,5]. This problem is avoided when 11-dehydro-$TxB_2$ in urine is measured. Moreover, measurements in blood only reflect point-to-point alterations, whereas sampling of urine includes 24-h integration. Urinary $TxB_2$ and 2,3-dinor-$TxB_2$ levels reflect renal and systemic formation of thromboxane under physiological conditions [6]. However, under pathophysiological circumstances associated with intense platelet activation, these metabolites might not accurately reflect renal and extrarenal thromboxane formation [6]. Hence, measurement of both 2,3-dinor-$TxB_2$ and 11-dehydro-$TxB_2$ has been proposed as a more accurate method to determine systemic thromboxane production [1,3,6,7].

The aim of this study was, therefore, to deter-

0378-4347/92/$05.00 

mine 11-dehydro-$TxB_2$, $TxB_2$, 2,3-dinor-$TxB_2$, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and 2,3-dinor-6-keto-$PGF_{1\alpha}$ in a single urine sample by gas chromatography–negative-ion chemical ionization mass spectrometry (GC–NICI-MS).

Since an extraction procedure has already been described for the aforementioned eicosanoids, except for 11-dehydro-$TxB_2$ [8], we optimized and extended this method to 11-dehydro-$TxB_2$.

Urinary 11-dehydro-$TxB_2$ is in equilibrium between the lactone (closed form) and its corresponding hydroxy acid (open form) [9,10]. This equilibrium can be forced either towards quantitative formation of the open form under alkaline conditions or towards the closed form under acidic conditions. The rate of the conversion is affected by pH and temperature [9].

The method described includes derivatization with pentafluorobenzyl bromide. For this reaction, the tautomers had to be converted quantitatively into either one of these forms in order to yield homogeneous reaction products.

The method was used to investigate the effects of the thromboxane synthase inhibitor Ridogrel (Janssen Pharmaceutica, Beerse, Belgium) [11,12] on the urinary excretion of eicosanoids after acute myocardial infarction.

## EXPERIMENTAL

### *Reagents and solvents*

11-Dehydro-[5,6,8,9,12,14,15(n)-$^3$H]$TxB_2$ was obtained from Amersham Buchler (Braunschweig, Germany). Its purity was proven by radio thin-layer chromatography (radio-TLC) (TLC plate: 19-channel silica gel, J. T. Baker, No. 70054) using the solvent system ethyl acetate–hexane–acetic acid–water (54:25:12:100, v/v). 11-Dehydro-$TxB_2$ was purchased from Cayman Chemical (Ann Arbor, MI, USA). 11-Dehydro-[18,18,19,19-$^2$H]$TxB_2$ was from Dr. C. O. Meese, Dr. Fischer-Bosch-Institut (Stuttgart, Germany). Its purity was proved by GC–NICI-MS after derivatization to its pentafluorobenzyl ester trimethylsilyl ether. All other reagents and solvents were of the highest purity available, as already described [8].

### *Biological samples*

For the determination of control levels, 24-h urine samples were collected over three consecutive days from six healthy, non-smoking, male volunteers, who were not allowed to take non-steroidal inflammatory drugs for at least ten days before urine collection. Urinary samples (24 h) were collected from patients ($n = 14$) with clinical and electrocardiographical evidence of acute myocardial infarction and considered to be candidates for thrombolytic therapy. Patients on antiplatelet or anti-inflammatory drug therapy were excluded. All patients received 100 mg of recombinant tissue-type plasminogen activator (rt-PA) over a period of 2 h. Just prior to the rt-PA infusion, patients received intravenously either placebo ($n = 7$) or 300 mg of Ridogrel ($n = 7$).

After collection the total volume was measured, and $^2H_4$labelled standards were added to 20-ml aliquots: 10 ng of $TxB_2$ and 6-keto-$PGF_{1\alpha}$ and 20 ng of 2,3-dinor-$TxB_2$ 11-dehydro-$TxB_2$ and 2,3-dinor-6-keto-$PGF_{1\alpha}$. Samples were stored at $-70$°C until analysis. Urinary levels of eicosanoids were expressed as picograms of eicosanoid per milligram of creatinine [8].

### *Extraction and purification procedure*

Determination of $TxB_2$, 2,3-dinor-$TxB_2$, 6-keto-$PGF_{1\alpha}$, 2,3-dinor-6-keto-$PGF_{1\alpha 3}$, $PGE_2$, $PGD_2$ and $PGF_{2\alpha}$ was done according to ref. 8, and modified as follows: methoximation was performed directly in urine (5 ml) with O-methylhydroxylammonium chloride at pH 8.6 (2.4 *M* phosphate buffer). The samples were applied to phenylboronic acid cartridges, prewashed with methanol and preconditioned with 10 $\mu M$ sodium hydroxide (pH 9). The cartridges were discarded after use. Prostaglandins (PG fraction) were eluted first with methanol–20 $\mu M$ sodium hydroxide (pH 9) (40:60, v/v) into 100 $\mu$l of formic acid, and subsequently thromboxanes (Tx fraction) were eluted using methanol–1.0 *M* sodium hydroxide (pH 14) (90:10, v/v) into 200 $\mu$l of formic acid. Both fractions (pH 3.5) were diluted with water and separately applied to $C_{18}$ cartridges, prewashed with methanol, water and acidified water (pH 3.5; formic acid). The cartridges were discarded after use.

After elution with ethyl acetate, both eluates were evaporated to dryness under nitrogen, and the residue was redissolved in ethyl acetate. The whole sample was applied to 19-channel silica gel TLC plates (J. T. Baker, No. 70054). TLC was carried out with the organic phase ethyl acetate–hexane–acetic acid–water (54:25:12:100, v/v; pH of organic phase 2.9; pH of aqueous phase 2.4). Corresponding zones of the eicosanoid-methoximes were localized by parallel development of 2 $\mu$g of a standard methoxime mixture. Visualization was achieved with 10% phosphomolybdic acid in ethanol. Eicosanoids were extracted from the silica gel with the TLC solvent and derivatized to their pentafluorobenzyl ester-trimethylsilyl ethers (PFBETMSE).

The whole procedure, except for the formation of PFBETMSE, was optimized by applying the open as well as closed form of the tritiated 11-dehydro-$TxB_2$ standard. Because this standard was available only in the closed form, aliquots of the standard were converted into the open form by allowing the solution to stand overnight at room temperature in 63 $\mu M$ ammonium bicarbonate adjusted to pH 8.6 with ammonium hydroxide [9]. The extent of reaction was evaluated via TLC and radioscanning of the plates with a Berthold Dünnschichtscanner. Subsequently urinary samples (5 ml) were spiked separately with 4000 Bq of the open and closed form of [$^3$H]-11-dehydro-$TxB_2$, respectively. These samples underwent the extraction procedure including TLC, whereas aliquots were taken after each separation step and subjected to liquid scintillation counting (scintillation cocktail: Readysafe, Beckman, Munich, Germany).

To convert the endogenous 11-dehydro-$TxB_2$ quantitatively into the closed form, two different methods were established and checked by radio-TLC. Urine was spiked with 4000 Bq of the open and closed forms. On the one hand, the urinary samples were acidified with 10% formic acid (pH 2) and left to react at 35°C. After 2 h, the samples were adjusted to pH 8.6 with 5 $M$ (*ca.* 1.5 ml) NaOH and extracted as already described. On the other hand, the dry eluates of the PG fraction from the $C_{18}$ cartridge were redissolved in 500 $\mu$l of ethyl acetate containing 10% formic acid. The samples were left to react at 35°C for 30, 45, 60 and 120 min. After evaporation to dryness, the samples were redissolved in 30 $\mu$l of ethyl acetate and subjected to TLC. The plates were analysed with a Berthold Dünnschichtscanner.

*Recovery, reproducibility, accuracy and data analysis*

For recovery studies, human urine (5 ml) was spiked with tritiated 11-dehydro-$TxB_2$. Additionally, urine was spiked with 5 ng of 11-dehydro-$TxB_2$ for reproducibility studies ($n$ = 5) and with 1, 2, 10, 15, and 20 ng for validation of the accuracy of the method ($n$ = 1). Calibration curves were prepared in the range from 40 pg/ml to 4 ng/ml of urine. To each sample, 5 ng of the deuterated compound were added.

All samples underwent the whole extraction procedure and GC–MS analysis. Data were evaluated with a calibration curve of standards that did not undergo the extraction procedure. Results were expressed as mean ± S.E.M. Statistical evaluation was performed using the Mann–Whitney $U$-test for unpaired observations.

*Gas chromatography–mass spectrometry*

A Hewlett-Packard 5890 gas chromatograph equipped with a cold injection system (Gerstel, Mühlheim, Germany was used. Chromatography was carried out using an Ultra 2 capillary column (12 m × 0.2 mm I.D., 0.33 $\mu$m film thickness), directly connected to the ion source of the mass spectrometer (MS 8230, Finnigan MAT, Bremen, Germany). Analysis was carried out using NICI with ammonia as the reagent gas. Registration and quantification of the $[M - PFB]^-$ ions were performed with multiple-ion detection. Detected masses were 511 for 11-dehydro-$TxB_2$ and 515 for the deuterated standard. The resolution was 1500 [8].

## RESULTS AND DISCUSSION

The aim of this study was to develop a method to determine 11-dehydro-$TxB_2$ together with 6-keto-$PGF_{1\alpha}$, 2,3-dinor-6-keto-$PGF_{1\alpha}$, $TxB_2$ and

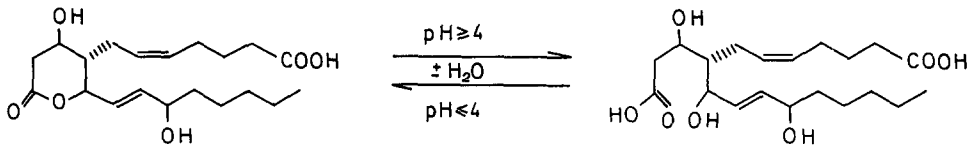


Fig. 1. pH-dependent tautomerism of 11-dehydro-$TxB_2$.

2,3-dinor-$TxB_2$ in a single urine sample to elucidate the eicosanoid profile after thromboxane synthase inhibition with Ridogrel. Because a method already exists for the analysis of the above-mentioned eicosanoids [8], except for 11-dehydro-$TxB_2$, we chose to adapt this method rather than to develop a separate or new method.

11-Dehydro-$TxB_2$ exhibits a pH-dependent equilibrium between the open and closed forms of its lactone ring (Fig. 1). A conversion into just one of these forms is possible either under acidic conditions to the closed form or under alkaline conditions to the open form. The rate of these conversions is strongly dependent on the pH and the temperature [9].

To validate the existing extraction procedure for both lactone forms, a $^3H$-labelled standard was used. Because the standard was available only in the closed form, a portion was transformed into the open form. Quantitative conversion was achieved when the sample was dried, redissolved in 63 $\mu M$ ammonium bicarbonate adjusted to pH 8.6 with ammonium hydroxide, and left to react overnight at room temperature. Efforts to adjust the pH to 8.6 using 2.4 $M$ phosphate did not result in quantitative conversion into the open form [9]. The extent of reaction was monitored by TLC, and radioscanning of the developed plates. The $R_F$ values were 0.15 for the open and 0.59 for the closed form.

Urinary samples were separately spiked with both of the lactone forms and underwent the extraction procedure [8].

Methoximation of $TxB_2$ and 2,3-dinor-$TxB_2$ resulted in the formation of 1,3-diols. These diols in turn were chemically bound to the boronate groups of a phenylboronic acid cartridge [8,13,14]. Whereas the open form of 11-dehydro-$TxB_2$ also possesses a 1,3-diol group (Fig. 1), the closed form does not. However, both tautomers were quantitatively retained by the cartridge (preconditioned with 10 $\mu M$ sodium hydroxide at pH 9), albeit both could be eluted in the PG fraction using methanol–20 $\mu M$ sodium hydroxide (pH 9) (40:60, v/v) up to 95 ± 2% ($n$ = 6), indicating only weak binding towards the boronate groups. The quantitative elution of $TxB_2$ and 2,3-dinor-$TxB_2$ from the cartridge was possible only by subsequent flushing with 10 ml of methanol–0.1 $M$ sodium hydroxide (pH 14) (90:10, v/v) [8].

11-Dehydro-$TxB_2$ in the PG fraction and submitted to the $C_{18}$-cartridge was eluted quantitatively as already described for the other prostaglandins.

The following TLC purification resulted in chromatograms exhibiting only one peak if the closed form of 11-dehydro-$TxB_2$ was applied to the extraction procedure but two peaks if the open form was applied (Fig. 2A and B). This experiment demonstrates that the ring begins to close during extraction.

These results show that it is not necessary to convert 11-dehydro-$TxB_2$ into just one form prior to the solid-phase extraction. However, because the original extraction procedure included a TLC step for further purification, and application of the open and closed ring forms results in different $R_F$ values, it is necessary to convert the endogenous compound into the closed form at least before TLC separation. Additionally, for subsequent esterification the presence of the closed form is a prerequisite.

Conversion into closed 11-dehydro-$TxB_2$ was tried by two different methods: by acidification of the urinary sample and by acidification of the $C_{18}$ eluate. When urinary samples spiked with the open form of [$^3H$]11-dehydro-$TxB_2$ were acidified to pH 2 with formic acid and left to react for 2 h at 35°C, the TLC chromatograms still

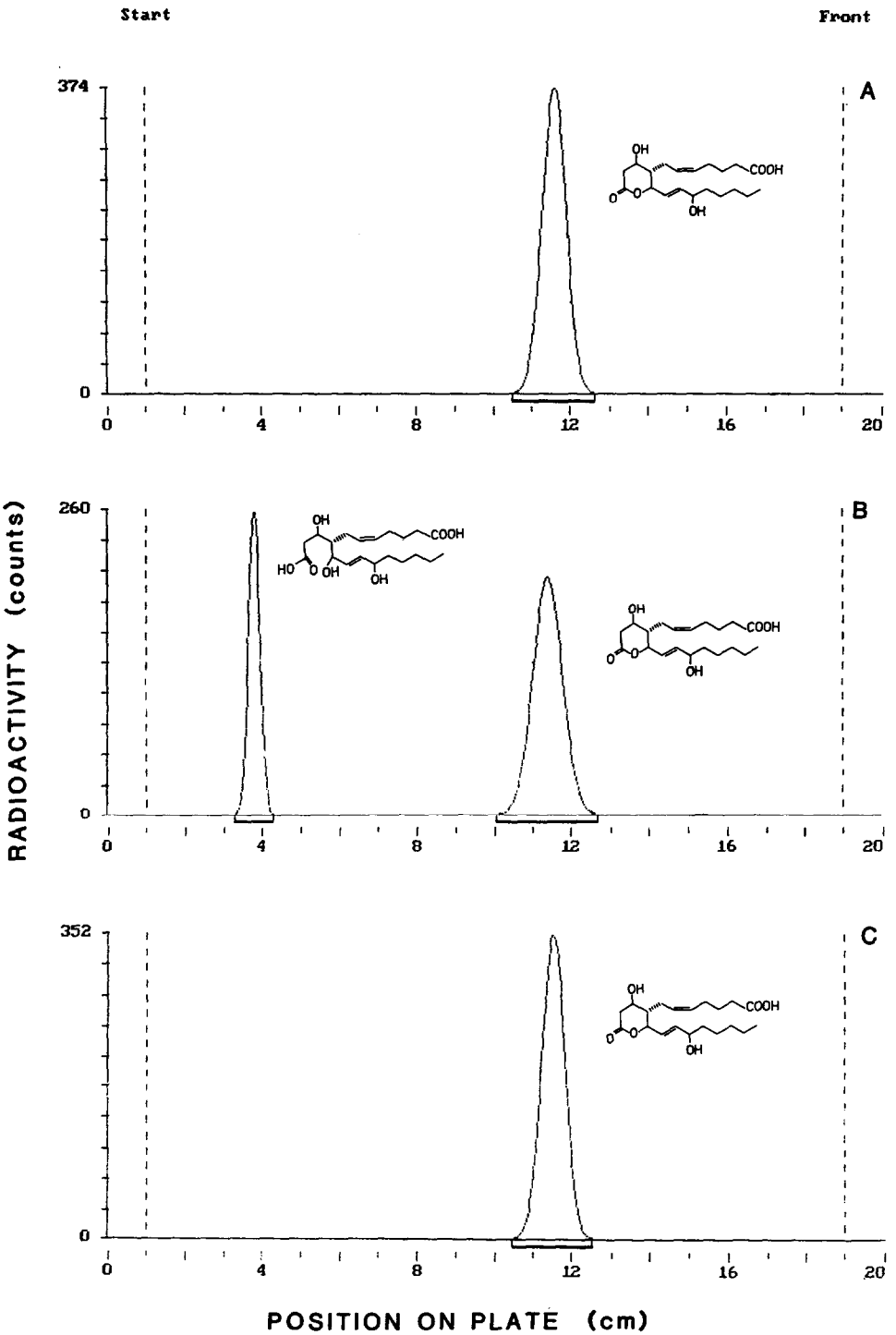


Fig. 2. Radio-TLC chromatograms of urine after the extraction and purification procedure. (A) Urine spiked with the closed form of 11-dehydro-TxB$_2$; (B) urine spiked with the open form; (C) urine spiked with the open form after passage through a $C_{18}$ cartridge and conversion into the closed form.

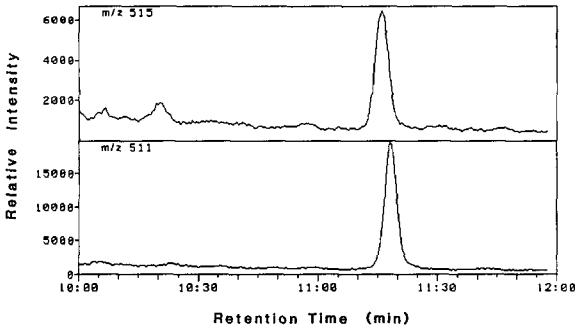


Fig. 3. GC–NICI-MS chromatogram of 11-dehydro-$TxB_2$ and its deuterated standard extracted from human urine (mass resolution 1500).

yielded two peaks. Because application of the closed form to the extraction procedure never resulted in two TLC peaks, it is assumed that the acidification of the urinary sample was not leading to a quantitative formation of cyclic 11-dehydro-$TxB_2$. However, when the dried eluates from the $C_{18}$ cartridge (PG fraction) were redissolved in ethyl acetate containing 10% formic acid and allowed to react at 35°C for 30, 45, 60 and 120 min, radio-TLC chromatograms of these samples showed that the open form was quantitatively closed after 45 min (Fig. 2C).

The TLC step yielded a recovery of *ca.* 85%. The overall recovery of the described method was 80%, which is within the range reported for the other prostaglandins [8]. A GC–NICI-MS chromatogram of an urinary extract is shown in Fig. 3. The difference in elution time for the deuterated standard and its endogenous analogue is assumed to be due to the isotope effect. This is in line with results of other authors [10,13].

TABLE I

ACCURACY OF THE METHOD

Concentration of 11-dehydro-$TxB_2$ found after addition of known amounts to human urine and subtraction of the blank value; urine sample size 5 ml; $n = 1$.

| Amount added (ng/ml) | Amount found (ng/ml) |
|---|---|
| 0.4 | 0.39 |
| 1 | 1.0 |
| 2 | 2.1 |
| 3 | 2.9 |
| 4 | 4.2 |

TABLE II

URINARY EXCRETION OF 11-DEHYDRO-$TxB_2$ FROM SIX HEALTHY MALE VOLUNTEERS ON THREE CONSECUTIVE DAYS

Values are given in pg/mg of creatinine resulting from a single analysis ($n = 1$).

| Volunteer | Day 1 | Day 2 | Day 3 |
|---|---|---|---|
| 1 | 1003 | 1027 | 1204 |
| 2 | 779 | 820 | 725 |
| 3 | 608 | 528 | 485 |
| 4 | 489 | 486 | 514 |
| 5 | 451 | 496 | 475 |
| 6 | 533 | 578 | 515 |

The reproducibility for the whole extraction procedure and GC–MS analysis was 2.5% ($n = 5$). Calibration curves from urine samples (range 40 pg/ml to 20 ng/ml) were linear with a correlation coefficient of 0.996. The detection limit was 10 pg/ml of urine. The accuracy of the method is given in Table I.

Control levels of 11-dehydro-$TxB_2$ measured in six healthy, male, non-smoking volunteers were in the range 450–1200 pg/mg of creatinine. Changes of 11-dehydro-$TxB_2$ levels observed in the urine of each volunteer during the three consecutive days were very low (Table II). The resulting mean (± S.E.M.) value was 662 ± 53 pg/mg of creatinine and is in good agreement with values published by Catella and FitzGerald [7]: 792 ± 119 pg/mg of creatinine for healthy normal volunteers. However, other investigators reported lower levels, *e.g.* Uedelhoven *et al.* [15] 332.6 ± 30.9, and Barrow *et al.* [16] 221 ± 18 pg/mg of creatinine for non-smokers.

Because the extraction procedure described earlier [8] was slightly modified, the method was proven for all other eicosanoids mentioned. No changes were observed for recovery, reproducibility, accuracy, purity or sensitivity for any of these eicosanoids.

Levels of 2,3-dinor-$TxB_2$ and 11-dehydro-$TxB_2$ in 24-h urine samples from patients with

myocardial infarction treated with thrombolytic therapy were 731 ± 94 and 1840 ± 354 pg/mg of creatinine, respectively (mean ± S.E.M., $n = 7$). Values ranged from 394 to 1030 pg/mg of creatinine for 2,3-dinor-$TxB_2$ and from 500 to 3500 pg/mg of creatinine for 11-dehydro-$TxB_2$. In patients receiving Ridogrel prior to thrombolytic therapy, significantly lower levels of 2,3-dinor-$TxB_2$ ($p < 0.001$) and 11-dehydro-$TxB_2$ ($p < 0.001$) were observed (243 ± 16 and 581 ± 52 pg/mg of creatinine, respectively, mean ± S.E.M., $n = 7$). These results confirm our previous findings that Ridogrel inhibits systemic thromboxane formation, and this observation is now extended to patients in whom extensive platelet activation occurred.

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