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Note

Determination of a new thromboxane A_2 receptor blocker in biological fluids by capillary gas chromatography with electron-capture detection

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4-[2-(4-Chlorophenylsulphonylamino)ethyl]phenylacetic acid (I, Fig. 1) has been characterized as a specific, selective and long-acting thromboxane A_2 (TXA_2) receptor antagonist. In rats and rabbits it inhibits the increase of arterial blood pressure or antagonizes the constriction of isolated arterial strips stimulated by U 46619^+ in a dose-related manner. U 46619 is regarded as the stable analogue of the cyclic prostaglandin endoperoxide PGH_2 and acts in a similar way to TXA_2. It also inhibits platelet aggregation induced by U 46619^+ or arachidonic acid [1–4]. For pharmacokinetic studies in humans we developed recently a simple, sensitive method for the determination of this compound. This method employs an internal standard (I.S., 4-[2-(4-chlorophenylsulphonylamino)ethyl)phenylpropanoic acid, II, Fig. 1), liquid–liquid extraction, derivatization and gas chromatography (GC) with electron-capture detection (ECD).

EXPERIMENTAL

Reagents

The reference standards (I and II) were synthesized in our own laboratories [5]. Analytical-grade reagents were used as supplied by the manufacturers. Hydrochloric acid in methanol was prepared by introducing hydrogen chloride gas into methanol (2–3% by weight).

Apparatus and GC conditions

The GC system consisted of a Hewlett-Packard Model 5710 A with capillary inlet system HP 18740 B, or a 5700 A Model without capillary inlet system, a ^{63}Ni

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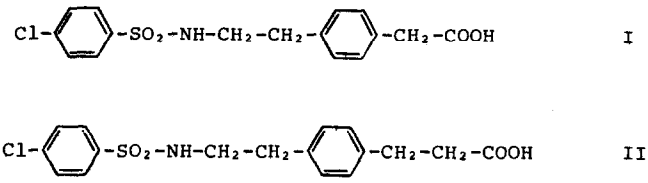


Fig. 1. Chemical structures of I and II.

electron-capture detector, an HP 7672 A automatic sampler (Hewlett-Packard, Böblingen, F.R.G.) and a Siemens Kompensograph III recorder (Erlangen, F.R.G.).

The measurements were carried out with a 10 m×0.53 mm I.D. mega-bore fused-silica column DB 17 (100% methylphenylpolysiloxane, film thickness 1.0 μm) from ICT Handelsgesellschaft (Frankfurt, F.R.G.). The operating conditions for routine analysis were: oven temperature, 260°C; injection port temperature, 280–300°C; detector temperature, 300°C; injection volume, 1 μl; carrier gas, hydrogen at 15 ml/min; make-up gas, 5% methane in argon at 30 ml/min. The total analysis time was ca. 8–10 min. Likewise it is possible to use a 30 m×0.53 mm I.D. mega-bore fused-cilica column DB 5 (95% dimethyl–5% diphenylpolysiloxane, film thickness 1.5 μm)) from ICT; the carrier gas was then hydrogen at 30 ml/min, and the make-up gas was 5% methane in argon at 10 ml/min.

Procedure

A 1-ml sample of plasma (serum), or 0.5 ml of urine, was mixed with 100 μl of I.S. (10 μg/ml in methanol for plasma samples, 100 μg/ml for urine samples). After the addition of 5 ml of diethyl ether and 50 μl of concentrated hydrochloric acid, the tubes were shaken in a tube rack on a reciprocating shaker for 15–20 min and centrifuged for 5 min at 4000 *g*. The organic phase was transferred to a 10-ml conical centrifuge tube and evaporated to dryness at 50°C under a stream of nitrogen. For derivatization the residue was taken up in 100–200 μl of methanolic hydrochloric acid and heated at 50°C for 15 min. After evaporation to dryness, the residue was dissolved in 200 μl of methanol. The solution was transferred to a sample vial and evaporated to dryness. Depending on the sample concentration, the residue was taken up in 25–1000 μl of methanol. The tubes were closed and vortexed, and 1 μl was injected into the GC system.

Calibration graphs were prepared by assaying plasma (urine) samples to which known amounts of standard (I) and I.S. (II) had been added. Peak-height ratios of I relative to II were plotted versus the added amounts of I.

For the experimental fit of the data-points to the regression line, three modes of regression are possible:

$y=a+bx$ (linear regression)
$y=ax^b$ (powerfit regression)
$y=a+bx+b_1x^2$ (non-linear regression)

where x=peak-height ratio and y=concentration of I in ng/ml. The coefficients of correlation of the different regression equations were routinely calculated to

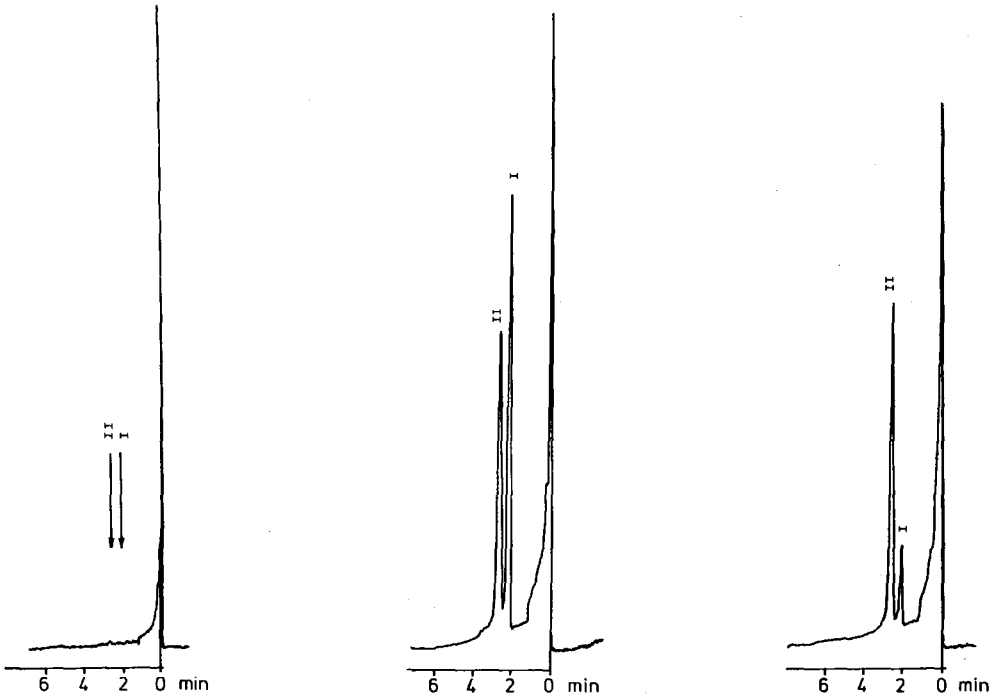


Fig. 2. Chromatogram of a drug-free control human plasma sample. Column: DB 17.

Fig. 3. Chromatogram of a human plasma sample spiked with 960 ng/ml I and 1000 ng/ml II. Column: DB 17.

Fig. 4. Chromatogram of an extract of a 1-ml human plasma sample of a volunteer taken 10 h after a single oral dose of 400 mg of I. The concentration is found to be 123.0 ng/ml I (1000 ng/ml I.S. Column: DB 17.

evaluate the fit of the calibration data to the regression line (>0.99). The function with the best correlation was used for the calibration of the sample concentrations.

RESULTS

Specificity

Drug-free control human plasma shows no significant peaks at the retention times of I or II when analysed by this method (Fig. 2).

Fig. 3 shows the chromatogram of an extract of 1 ml of human plasma spiked with 960 ng/ml I and 1000 ng/ml I.S.

Fig. 4 shows the chromatogram of an extract of 1 ml human plasma taken 10 h after an oral dose of 400 mg of I.

The identity of I and II in the derivatized form as the methyl ester was proved and confirmed with gas chromatography–mass spectrometry.

TABLE I

PLASMA DATA FOR PRECISION AND ACCURACY FROM A 2 μg/ml POOL OF COMPOUND I

Work-up	Concentration (mean ± S.D.) (μg/ml)	R.S.D. (%)
1	1.91 ± 0.02	0.77
2	1.93 ± 0.04	2.00
3	2.20 ± 0.03	1.57
4	2.09 ± 0.11	5.36
5	2.16 ± 0.06	2.57
6	2.05 ± 0.03	1.65
Mean	2.05 ± 0.12	5.92

Accuracy and precision

To determine the between-run and the within-run precision and accuracy, a plasma pool was spiked with 2 μg/ml I. With six different calibration curves six samples from this pool in each case were analysed, giving a total number of 36 samples. The values of the mean and the S.D. calculated from the six measurements of each work-up represent the within-run accuracy and precision. The between-run accuracy and precision are indicated by the mean and S.D. of the six within-run calculations (Table I). This procedure was used analogously for the analysis of urine samples spiked with 25 μg/ml I (Table II).

Recovery

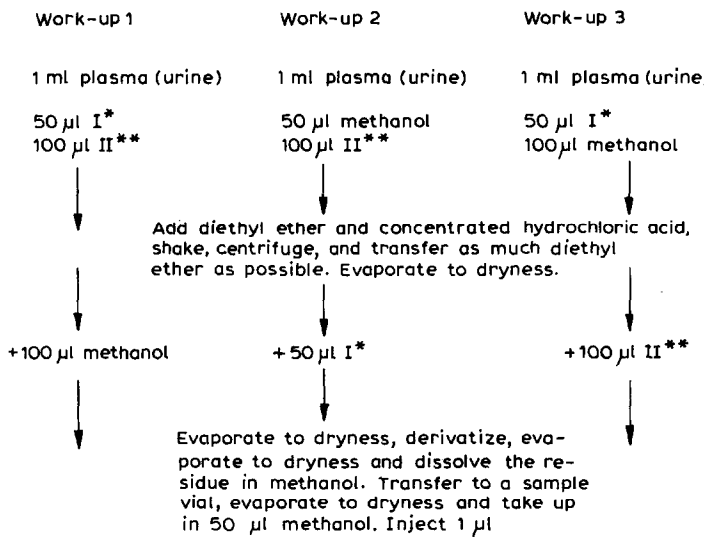
The recovery of I and II in plasma and urine was studied. The procedure used for this study is shown in Fig. 5.

Work-up 2 represents 100% recovery of compound I and work-up 3 100% recovery of the I.S. Each work-up was repeated ten times. For each set, mean peak heights have been calculated and percentage recovery of I and II determined by comparing samples 1 and 2 and samples 1 and 3, respectively. Recovery from

TABLE II

URINE DATA FOR PRECISION AND ACCURACY FROM A 25 μg/ml POOL OF COMPOUND I

Work-up	Concentration (mean ± S.D.) (μg/ml)	R.S.D. (%)
1	21.11 ± 0.26	1.24
2	21.33 ± 0.51	2.37
3	23.92 ± 0.56	2.34
4	24.36 ± 0.34	1.41
5	25.12 ± 0.20	0.80
6	21.13 ± 0.29	1.37
Mean	22.83 ± 1.74	7.61



* Methanolic standard solution: 10 µg/ml for plasma samples, 100 µg/ml for urine samples

** Methanolic I.S. solution: 10 µg/ml for plasma samples, 50 µg/ml for urine samples

Fig. 5. Procedure for recovery study.

plasma samples was 63.4% for I and 61.3% for I.S. Recovery from urine samples was 86.1% for I and 87.8% for I.S.

Detection limit

Ten individual serum standards and twelve individual urine standards in the lower range of the calibration curve (30, 50 and 100 ng/ml for serum; 50, 100 and 150 ng/ml for urine) were assayed, and the value of the mean and the S.D. were calculated. The limit of detection (DL) was obtained according to the following equation:

$$\mathrm{DL} = X + \frac{3sn + 3sn - 1 + \ldots + 3s}{n}$$

where X is the mean value of the blank signal, sn is the S.D. of an individual concentration range assayed, and n is the number of concentration ranges assayed. Based on reference calibration curves of 0–500 ng/ml for serum and 0–200 ng/ml for urine, the DL for compound I was found to be 8.2 ng/ml in serum and 29.4 ng/ml in urine.

CONCLUSION

This paper reports a sensitive and selective GC–ECD method for the detection of a new thromboxane A_2 receptor blocker in plasma (serum) and urine samples. Diethyl ether was found to be an efficient solvent for single-step plasma (urine) extraction, providing low-level drug quantitation without any interference from

biological fluid constituents. The method permits the routine analysis of the large number (up to 100 a day) of samples required for pharmacokinetic studies.

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