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Note

Determination of the anti-anginal drug 6-chloro-2-pyridylmethyl nitrate in plasma and urine by fused-silica open-tubular column gas chromatography with electron-capture detection

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6-Chloro-2-pyridylmethyl nitrate (CPMN) possesses good anti-anginal activity [1,2] and is currently undergoing clinical trials. The disposition of this drug in rats and dogs was reported in previous papers [3,4].

This paper describes simple and sensitive methods for the determination of CPMN in human, dog and rat plasma and human urine.

EXPERIMENTAL

Materials

CPMN (Fig. 1) was synthesized and supplied by the Department of Organic Chemistry, Research Laboratories, Fujisawa Pharmaceutical (Osaka, Japan). 3,4-Dichloroacetophenone and 3,5-dichloroanisole as internal standards were purchased from Aldrich (Milwaukee, WI, U.S.A.) and glyceryl trinitrate (GTN) from Nippon Kayaku (Tokyo, Japan). Toluene was of spectrophotometric grade and the other reagents and solvents were of extra-pure grade.

Standard solution of CPMN and GTN were prepared by dissolving them in a 30% solution of methanol in water and diluting to appropriate concentrations. The internal standards were dissolved in toluene.

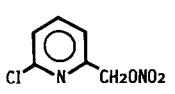

Fig. 1. Structure of 6-chloro-2-pyridylmethyl nitrate (CPMN).

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Gas chromatography (megabore column)

A Hewlett-Packard 5710A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) was equipped with a ^{63}Ni electron-capture detector, a Model 7672A automatic sampler (Hewlett-Packard) and a Model 3392A integrator (Hewlett-Packard). A 10 m × 0.53 mm I.D. fused-silica open-tubular megabore column (HP-5, Hewlett-Packard) with a coating thickness of 2.65 μm was used. The injection port and detector were operated at 150 and 250°C, respectively, and the column oven was maintained at 125°C. The flow-rate of the carrier gas [argon–methane (95:5, v/v)] was 24 ml/min. Under these conditions, CPMN, GTN and the internal standard (3,5-dichloroanisole) eluted with retention times of 2.8, 2.5 and 1.5 min, respectively (see Figs. 2 and 4).

Gas chromatography (capillary column)

A Hewlett-Packard 5890A gas chromatograph was equipped with an electron-capture detector, an automatic sampler and an integrator. A 25 m × 0.32 mm I.D. 5% phenylmethylsilicone-coated fused-silica capillary column (Ultra 2, Hewlett-Packard) with a coating thickness 0.52 μm was used.

The sample was introduced into a splitless injector maintained at 140°C. The temperature of the column oven was operated at 80°C for 1 min, followed by temperature programming at 10°C/min up to 125°C, held for 10 min, then at 70°C/min up to 240°C, held for 3 min. The detector was maintained at 300°C. Helium was used as the carrier gas at a head pressure of 60–95 kPa. The flow-rate of the make-up gas [argon–methane (95:5, v/v)] was 30 ml/min. Under these conditions, CPMN and the internal standard (3,4-dichloroacetophenone) eluted with retention times of 10.1 and 11.2 min, respectively (see Fig. 3).

Extraction procedure

To a 10-ml centrifuge tube were added 1.5 ml of plasma sample or human urine, 150 μl of 30% methanol in water and 2.5 ml of distilled *n*-pentane. The tube was shaken for 3 min and centrifuged at 2000 *g* for 5 min. A 2-ml volume of the organic phase was transferred into a 5-ml Reacti-Vial (Pierce, Rockford, IL, U.S.A.), and then 100 μl of toluene internal standard solution were added. The solvent was evaporated in vacuo at room temperature to a volume of 100 μl. The final volume after evaporation was not critical, as all calculations were based on the peak height of the internal standard. A 3-μl aliquot of the solution was injected into the gas chromatograph.

All glassware was siliconized and heated at approximately 100°C.

Quantitation

The procedure was standardized by analysing blank plasma or urine to which had been added 150 μl of CPMN or GTN standard solutions instead of 150 μl of 30% methanol solution as in the extraction procedure. Peak-height ratios of CPMN to the internal standard and GTN to the internal standard were used to establish the calibration graphs.

Precision and accuracy

Blank plasma samples containing CPMN or GTN and urine samples containing CPMN were carried through the above procedure.

Stability

To study whether the storage of the plasma sample affected the plasma concentration of CPMN, spiked plasma samples containing 5 ng/ml CPMN were frozen at −20°C. The concentrations of CPMN in samples frozen for one month were determined.

Animal study

Male beagle dogs (Japan Experimental Dogs and Monkeys) weighing 10–12 kg and male Sprague–Dawley rats (CLEA Japan, Tokyo, Japan) weighing 200–260 g were used. The animals were given an intravenous dose of 1 mg/kg CPMN or GTN. The plasma samples were stored at −20°C until analysed.

RESULTS AND DISCUSSION

Separation

Typical chromatograms for CPMN obtained from rat, dog and human plasma samples are shown in Figs. 2 and 3. As shown, the background peaks of the blank rat plasma, dog plasma and human plasma were completely separated from the peaks of CPMN and the internal standard. There was no interference at the retention times of CPMN and the internal standard. Although not shown here,

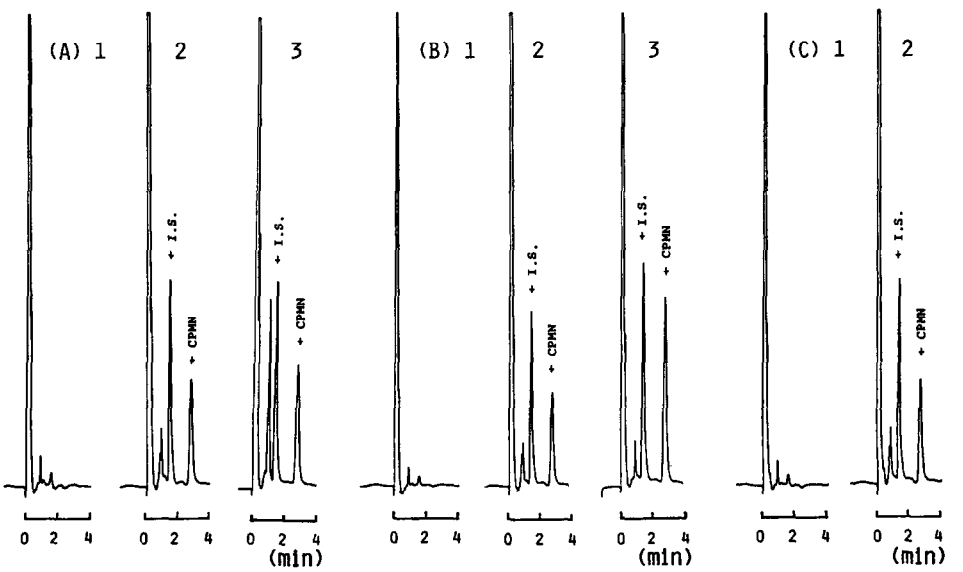

Fig. 2. Chromatograms (megabore column) of (A) rat plasma, (B) dog plasma and (C) human plasma. 1 = Blank plasma; 2 = blank plasma samples spiked with 5 ng/ml CPMN; 3 = plasma collected from a rat and a dog after an intravenous dosing of 1 mg/kg CPMN. I.S. = internal standard (3,5-dichloroanisole; 1 μg/ml).

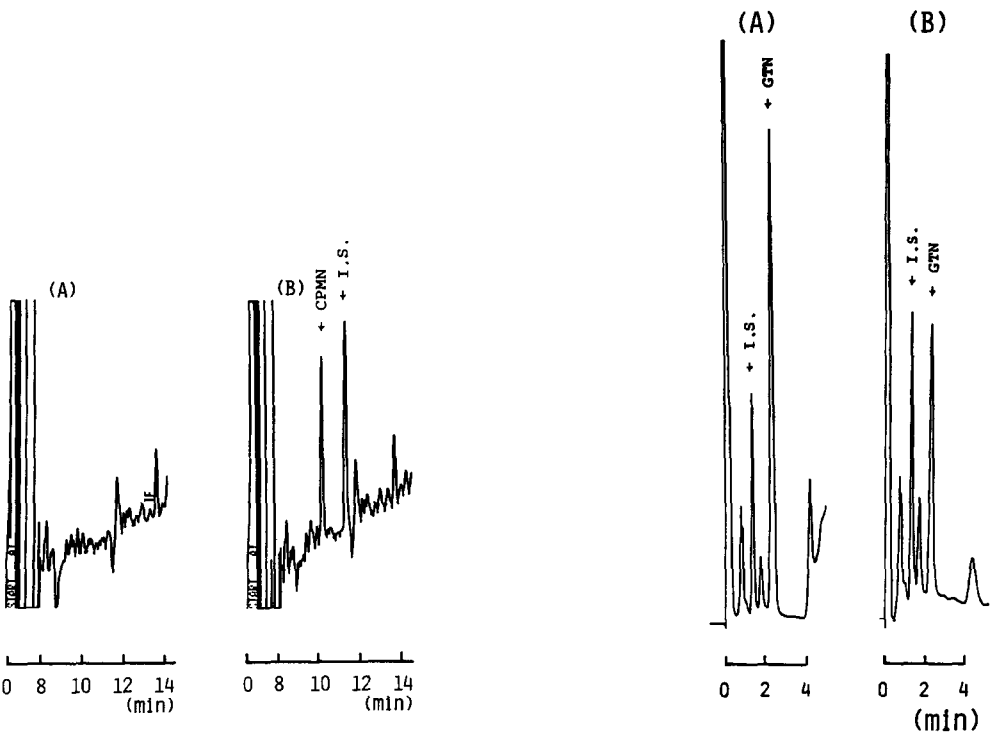

Fig. 3. Chromatograms (capillary column) of (A) blank human plasma and (B) human plasma (containing 0.5 ng/ml CPMN) treated according to the method described under Experimental. I.S. = internal standard (3,4-dichloroacetophenone; 2 ng/ml).

Fig. 4. Chromatograms (megabore column) of (A) rat blank plasma spiked with 50 ng/ml GTN and (B) plasma collected from a rat after an intravenous dosing of 1 mg/kg GTN. I.S. = internal standard (3,5-dichloroanisole; 1 μg/ml).

there were no interfering peaks with that of CPMN in the chromatograms obtained from human urine. For GTN quantitation (Fig. 4), the background peaks of blank rat plasma were separated from the peak of GTN as for CPMN.

Calibration graph

Typical calibration graphs for human, dog and rat plasma are given in Table I. The calibration graphs show good linearity in each range. The limit of determination of CPMN in plasma samples was 0.1 ng/ml with the megabore column and 0.05 ng/ml with the capillary column.

Reproducibility and stability

The reproducibility was evaluated by performing triplicate analyses of spiked plasma and urine samples. The results are given in Table II. The coefficients of variation (C.V.) were less than 7% and the actual concentrations of CPMN measured by GC ranged from 96 to 106% for all plasma and urine samples, except for lower concentrations in human urine. This GC method for the determination of

TABLE I

TYPICAL CALIBRATION GRAPHS FOR PLASMA AND URINE

Sample	Concentration range (ng/ml)	Slope	Intercept	Correlation coefficient
Megabore column				
CPMN: Human plasma	0.1–25	0.1627	0.0085	0.9996
Dog plasma	0.1–25	0.1575	−0.0068	0.9997
Rat plasma	0.1–25	0.2423	0.0238	0.9990
GTN: Rat plasma	0.25–50	0.04557	0.0456	0.9999
Capillary column				
CPMN: Human plasma	0.05–2.5	1.1477	0.0000	0.9998
Human urine	0.05–2.5	5.6098	0.1870	0.9992

TABLE II

REPRODUCIBILITY OF THE DETERMINATION OF CPMN AND GTN IN PLASMA AND URINE

Sample	Actual concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Percentage of actual concentration	Coefficient of variation (%)
Megabore column				
CPMN: Human plasma	0.5	0.49 ±0.012	98	2.4
	5	4.81 ±0.16	96	3.3
Dog plasma	0.5	0.53 ±0.015	106	2.8
	5	5.04 ±0.33	101	6.5
Rat plasma	0.5	0.51 ±0.015	102	2.9
	5	5.30 ±0.22	106	4.2
GTN: Rat plasma	5	5.22 ±0.080	104	1.5
	10	10.4 ±0.11	104	1.1
Capillary column				
CPMN: Human plasma	0.05	0.049 ±0.0015	96	3.1
	0.25	0.25 ±0.012	100	4.8
	1	0.99 ±0.075	99	7.6
Human urine	0.25	0.23 ±0.0058	92	2.5
	2.5	2.57 ±0.16	103	6.3

CPMN in plasma and urine thus provides good accuracy and precision. CPMN was stable in human, dog and rat plasma at −20°C for up to one month.

Plasma concentration in animals

Plasma concentrations of the unchanged drug in rats and dogs after an intravenous administration are shown in Fig. 5.

CONCLUSION

The quantitation of CPMN in human plasma and urine using a capillary column was possible down to 0.05 ng/ml using 1.5 ml of plasma or urine. By using a

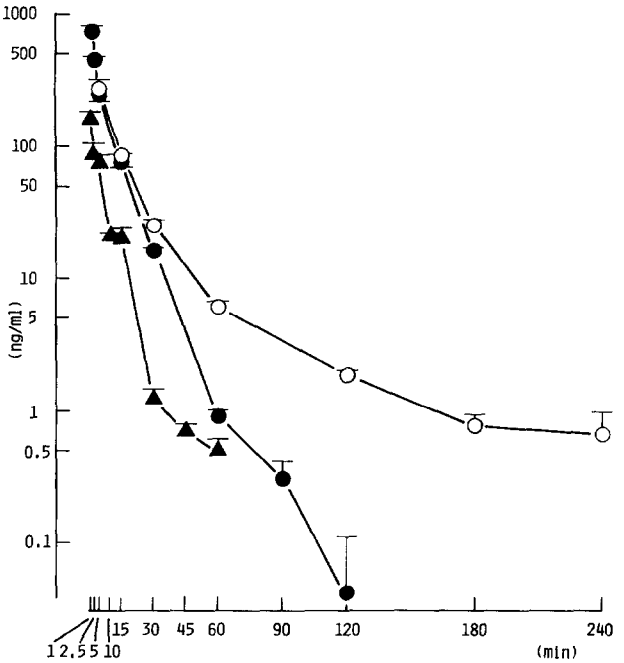

Fig. 5. Plasma concentrations of the unchanged drug after intravenous (1 mg/kg) bolus injection of CPMN to (●) rats and (○) dogs or (▲) GTN to rats. The vertical bars represent the standard deviations for three animals.

megabore column, quantitation was possible down to 0.1 ng/ml. Moreover, GTN could be determined by this technique (down to 0.25 ng/ml) in rat plasma. These methods are simple and relatively sensitive, and we have mainly used them for pharmacokinetic studies in animals [3,4]. We believe that they are adequate for determining CPMN in human plasma in clinical trials.

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