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DETERMINATION OF THE TWO DINITRATE METABOLITES OF NITROGLYCERIN IN HUMAN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

This paper describes a sensitive method for the specific determination of 1,2-glyceryl dinitrate and 1,3-glyceryl dinitrate as metabolites of nitroglycerin at concentrations down to 250 pg/ml plasma. After addition of a known amount of 2-isosorbide mononitrate as internal standard, plasma is introduced onto an Extrelut cartridge and the compounds of interest are eluted with dichloromethane. The glyceryl dinitrates are then quantitated by capillary gas chromatography with electron-capture detection.

INTRODUCTION

Nitroglycerin (glyceryl trinitrate, GTN) is a potent vasodilator agent. Both 1,2- and 1,3-glyceryl dinitrate (1,2- and 1,3-GDN) have been shown to be the major metabolites of GTN [1]. Several methods have already been proposed for their quantitative assay in biological fluids.

Two gas chromatographic (GC) methods that use electron-capture detection (ECD) have been published [2,3]. Wu et al. [2] described a packed-column GC method, but they detected a mixture of dinitrates. Recently, Noonan et al. [3] developed a method using a capillary column for the simultaneous determination of GTN and its dinitrate metabolites: they reported a coefficient of variation (C.V.) of 3.8% at 1.0 ng/ml for 1,2- and 1,3-GDN.

Several high-performance liquid chromatographic (HPLC) methods have been reported for the analysis of GTN and its metabolites [4–6], but two of them lack detectability. The third method, published by Woodward et al. [6], involved the use of a thermal energy analyser detector with a limit of detection for 1,2- and 1,3-GDN of 250 pg/ml in plasma.

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Two sensitive methods for determination of GTN, 1,2- and 1,3-GDN in plasma were based on GC combined with negative-ion chemical ionization mass spectrometry (MS) [7,8]; they used capillary columns and had a sensitivity between 6 pg/ml [7] and 1000 pg/ml [8] for 1,2- and 1,3-GDN. A sensitive method for the assay of unchanged GTN in plasma has recently been developed in our laboratories [9]. Using capillary GC with ECD, this method can measure concentrations down to 50 pg/ml. The extraction procedure elaborated for GTN, however, was not convenient for 1,2- and 1,3-GDN.

This paper describes a method, practicable in all laboratories and more easily available than GC–MS, that uses capillary GC with ECD for the quantitative determination of 1,2- and 1,3-GDN in human plasma. Its limit of quantification is 250 pg/ml 1,2- or 1,3-GDN in plasma.

EXPERIMENTAL

Chemicals and reagents

The two dinitrate metabolites (1,2- and 1,3-GDN) of GTN were provided by Société Nationale des Poudres et Explosifs (Vert-le-Petit, France) as a 1% solution in ethanol. The internal standard, 2-isosorbide mononitrate (2-ISMN), was supplied by Sanol (Monheim, F.R.G.). Dichloromethane and hexane (Pestipur, SDS, Peypin, France) were of analytical grade. The aqueous solutions of glyceryl dinitrates and internal standard were stored at 4°C and prepared freshly every month. The extraction columns used were pre-packed glass columns (Extrelut 1-15371; Merck, Darmstadt, F.R.G.).

Equipment

A Hewlett-Packard Model 5880 A gas chromatograph equipped with a computing integrator, an electron-capture detector and a 50 m × 0.32 mm I.D. fused-silica capillary column coated with methyl silicone (OV-1) (Hewlett-Packard No. 19091 A, Option 115) was used. A Chrompack On-Column injector was set up and continually cooled with air. Helium was used as carrier gas at a flow-rate of 1.5 ml/min with an inlet pressure of ca. $3.5 \cdot 10^5$ Pa; the flow-rate of auxiliary gas (argon–methane, 90:10) to the detector was 30 ml/min. The column temperature was held at 30°C initially for 0.1 min, then raised at 50°C/min to 135°C with an isothermal hold for 8 min at 135°C; to wash out plasma residues, the column temperature was held at 250°C for 6 min. The detector was set at 220°C.

A 1-m deactivated vitreous silica tube was used as a precolumn to prevent peak splitting. The precolumn was connected to the injector and to the analytical column by zero-dead-volume unions. Every 50 injections the precolumn had to be changed. To prevent adsorption, the glassware was pretreated by immersion in toluene containing hexamethyldisilazane, trimethylchlorosilane and pyridine (1%, v/v each) for 15 min and subsequently rinsed with methanol. Then the glassware was washed for 30 min in an ultrasonic bath filled with methanol.

Extraction and gas chromatography

Internal standard solution (25 μl, corresponding to 2500 pg of 2-ISMN) was measured into a 10-ml glass centrifuge tube, to which 1 ml of plasma was added. The tubes were shaken for a few seconds on a Vortex mixer.

The sample was introduced onto an Extrelut 1 cartridge. After ca. 6 min of equilibration, the glyceryl dinitrates and the internal standard were directly eluted, without washing, with two 3-ml volumes of dichloromethane. After evaporation of the eluate to dryness under nitrogen at 40°C, the residue was dissolved in 100 μl of hexane. A 2-μl portion of the hexane phase was injected onto the chromatographic column, using a syringe with a fused-silica capillary needle.

Calibration curves

Calibration samples were prepared by introducing a suitable volume of an aqueous solution of 1,2-GDN and 1,3-GDN (corresponding to between 250 and 5000 pg) into 10-ml glass centrifuge tubes containing 1 ml of plasma and a constant amount of internal standard (2500 pg). The calibration curves were obtained from the ln–ln plot of the peak-height ratios versus the plasma concentrations. The equations of the curves were estimated by the least-squares method using ln–ln regression after subtracting the blank value. The calibration curves were obtained every day.

Study in humans

Four subjects received on three different days 240 μg of 1,2-GDN, 240 μg of 1,3-GDN and 300 μg of GTN by intravenous infusion at a constant rate over 30 min. Blood samples were collected just before and at 15 and 30 min during the infusion. A last sample was collected at 45 min, i.e. 15 min after the end of the infusion. After centrifugation, plasma was removed and stored at −80°C until analysis.

RESULTS AND DISCUSSION

On-column injection

Initial attempts to use capillary GC with a splitless injector were unsuccessful: after seventeen successive injections of the same 1,2- and 1,3-GDN solution containing 50 pg/μl, the C.V.s calculated from the respective peak heights were 59% for 1,3-GDN and 54% for 1,2-GDN. These coefficients decreased to 10% in the case of on-column injection. When 2-ISMN was used as internal standard, the C.V.s calculated from the peak-height ratio were lower than 10%.

Calibration curves

Some variation in the standard curves obtained on separate days was noticed, which was the reason why they were obtained daily.

Within-day precision

The within-day precision of the method was checked by determining six plasma samples spiked with different concentrations of 1,2-GDN and 1,3-GDN. The results obtained with the procedure described above are given in Tables I and II.

TABLE I

WITHIN-DAY PRECISION OF THE ASSAY AND RECOVERY OF 1,2-GDN IN SPIKED PLASMA SAMPLES
1 ng/ml is equivalent to 5.49 nmol/l.

	0.25 ng/ml added		1.50 ng/ml added		3.00 ng/ml added		5.00 ng/ml added	
	Found (ng/ml)	Recovery (%)	Found (ng/ml)	Recovery (%)	Found (ng/ml)	Recovery (%)	Found (ng/ml)	Recovery (%)
	0.22	88.0	1.60	106.7	3.14	104.7	4.72	94.4
	0.25	100.0	1.53	102.0	3.00	100.0	5.00	100.0
	0.28	112.0	1.54	102.7	2.92	97.3	4.96	99.2
	0.27	108.0	1.63	108.7	2.87	95.7	4.77	95.4
	0.27	108.0	1.49	99.3	2.98	99.3	4.69	93.8
	0.25	100.0	1.49	99.3	3.04	101.3	4.99	99.8
Mean	0.26	102.7	1.55	103.1	2.99	99.7	4.86	97.1
C.V. (%)	8.42		3.72		3.15		2.95	
Mean recovery ± S.D. (%)				100.7 ± 5.40				

Plasma interference

Fig. 1 shows the chromatograms of an extract of human blank plasma (1 ml) and of an extract of human plasma (1 ml) spiked with 2.5 ng of 1,2-GDN and 1,3-GDN, and internal standard. No interference from normal plasma components was recorded. This extraction procedure using an Extrelut cartridge was preferred to a conventional solvent extraction, which presented more severe interferences.

Selectivity

GTN and its 1- and 2-glyceryl mononitrate metabolites were injected under the same conditions as 1,2- and 1,3-GDN. These compounds were detected with retention times of 3.35 and 3.61 min for the mononitrates, and 5.82 min for GTN. The retention times of 1,3- and 1,2-GDN were 4.96 and 5.09 min, respectively, and were clearly separated from GTN and the mononitrate metabolites. Fig. 2 shows the chromatogram of a mixture of GTN and its four metabolites.

Application

The present method was used to determine the plasma concentrations of the glyceryl dinitrates after intravenous administration of 1,2-GDN, 1,3-GDN and

TABLE II

WITHIN-DAY PRECISION OF THE ASSSAY AND RECOVERY OF 1,3-GDN IN SPIKED PLASMA SAMPLES
1 ng/ml is equivalent to 5.49 nmol/l.

	0.25 ng/ml added		1.50 ng/ml added		3.00 ng/ml added		5.00 ng/ml added	
	Found (ng/ml)	Recovery (%)	Found (ng/ml)	Recovery (%)	Found (ng/ml)	Recovery (%)	Found (ng/ml)	Recovery (%)
	0.23	92.0	1.75	116.7	3.00	100.0	4.81	96.2
	0.23	92.0	1.63	108.7	2.79	93.0	5.22	104.4
	0.29	116.0	1.70	113.3	2.76	92.0	5.00	100.0
	0.27	108.0	1.65	110.0	2.60	86.7	4.94	98.8
	0.26	104.0	1.50	100.0	2.75	91.7	4.71	94.2
	0.25	100.0	1.48	98.7	2.82	94.0	5.04	100.8
Mean	0.26	102.0	1.62	107.9	2.79	92.9	4.95	99.1
C.V. (%)	9.20		6.67		4.6		3.62	
Mean recovery ± S.D. (%)				100.5 ± 8.2				

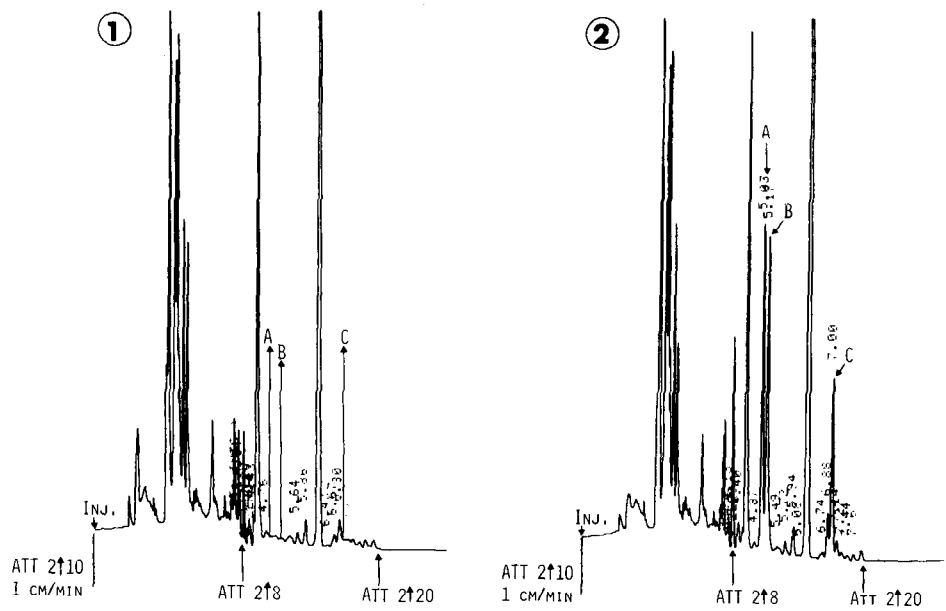

Fig. 1. Examples of chromatograms. (1) Human plasma blank (extract of 1 ml of plasma); (2) the same plasma spiked with 2.5 ng of 1,3-GDN (A), 2.5 ng of 1,2-GDN (B) and 2.5 ng of internal standard (C).

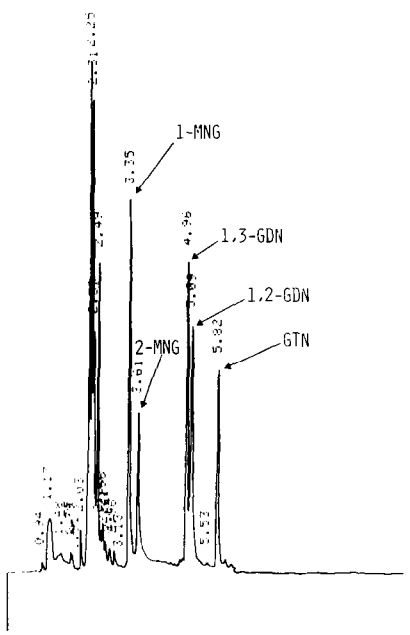

Fig. 2. Chromatogram of a mixture of 1- and 2-glyceryl mononitrate, 1,2- and 1,3-glyceryl dinitrate and glyceryl trinitrate (nitroglycerin).

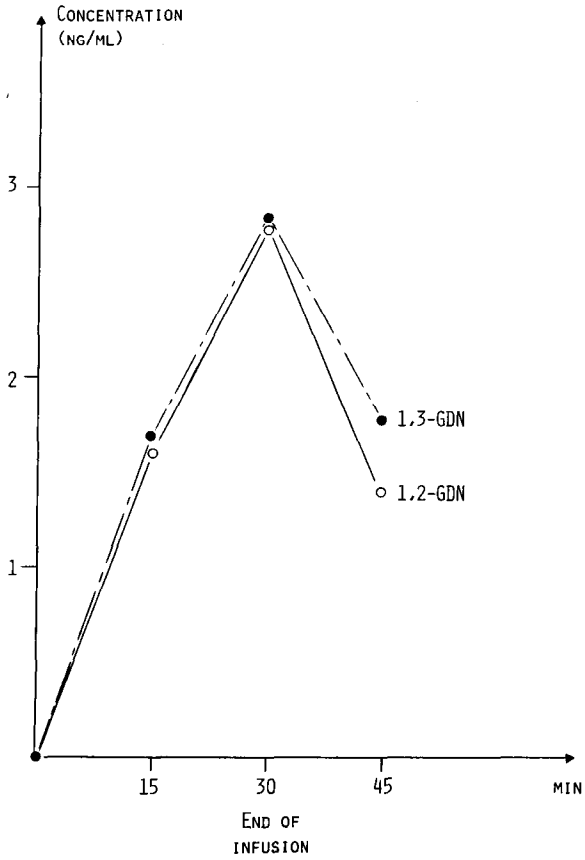

Fig. 3. Average plasma concentrations of glyceryl dinitrates obtained in four subjects after administration of 240 μg of 1,2-GDN (○) and 240 μg of 1,3-GDN (●) by intravenous infusion at a constant rate over 30 min.

GTN to four healthy volunteers on three different days. After administration of GTN, the corresponding plasma concentrations of parent drug (GTN) have been also determined [9].

Fig. 3 shows the average curves of 1,2-GDN and 1,3-GDN obtained from the plasma of four subjects given, 240 μg of 1,2-GDN and 240 μg of 1,3-GDN, respectively. Fig. 4 shows the average curves of 1,2-GDN and GTN obtained from plasma of four subjects given an equimolar dose of 300 μg of GTN; 1,3-GDN was not detected after administration of GTN.

CONCLUSION

The proposed capillary GC technique permits the quantitative assay of 1,2-GDN and 1,3-GDN in human plasma after administration of each of the two glyceryl dinitrate metabolites, and was applied for the determination of 1,2-GDN as a metabolite of GTN after administration of GTN.

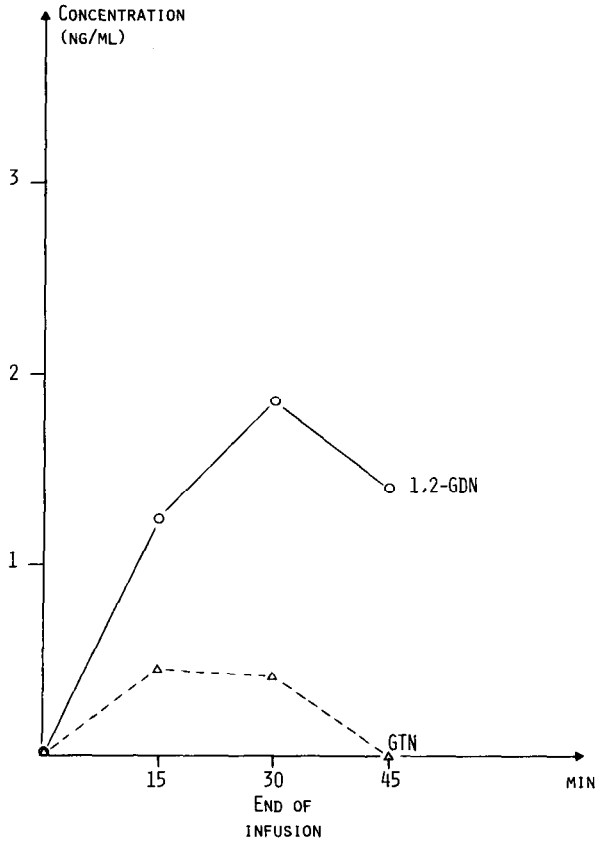

Fig. 4. Average plasma concentrations of 1,2-GDN and GTN obtained in four subjects after administration of 300 μg of GTN by intravenous infusion at a constant rate over 30 min. (○) 1,2-GDN; (△) GTN.

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