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QUANTITATIVE DETERMINATION OF NITROGLYCERIN IN HUMAN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A sensitive method for the selective determination of nitroglycerin at concentrations down to 50 pg/ml in human plasma is described. After the addition to plasma of a known amount of butane-1,2,4-triol trinitrate as internal standard, both compounds are extracted into hexane. Nitroglycerin is then quantitated by capillary gas chromatography with electron-capture detection.

INTRODUCTION

Nitroglycerin (1,2,3-propanetriol trinitrate, GTN) is a coronary dilator agent. Several methods have already been proposed for its quantitative assay in biological fluids.

Some gas chromatographic (GC) methods using packed columns and electron-capture detection (ECD) have been described [1—5]. Rosseel and Bogaert [1] achieved a sensitivity of 0.5 ng/ml using ethyl acetate extraction, but 5-ml plasma aliquots are required. Their technique was modified by Yap et al. [2] who used a laborious hexane extraction of plasma and reported a sensitivity of 0.1 ng/ml with about a 20% error. Two other GC techniques have been described but they require several millilitres of plasma and their sensitivity is poor [3, 4]. Wu et al. [5] developed two methods for the quantitation of GTN and its metabolites in human plasma using pentane or ethyl acetate as extraction solvent; the limit of sensitivity for GTN using their method is 50 ± 8.2 pg/ml. This sensitivity was also claimed by Rey et al. [6] and Kühn et al. [7] using capillary GC.

A high-performance liquid chromatographic method [8] has been published

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for the separation of GTN and its metabolites using a thermal energy analyser detector with a limit of sensitivity for GTN of 500 pg/ml of blood.

Gerardin et al. [9] described a gas chromatographic—mass spectrometric (GC—MS) method using positive-ion detection in electron-impact mode, which permits determination of GTN in plasma at concentrations down to 50 pg/ml with the ^{15}N-labelled compound as internal standard.

In the last two years, sensitive methods for the determination of GTN in plasma have been developed using GC—MS with chemical ionization in the negative mode [10—14]; several authors used capillary columns [10—12]. The GC—MS methods cited have a sensitivity between 6 pg/ml [13] and 100 pg/ml. Isosorbide dinitrate [1—3], metadinitrobenzene [4, 6], *o*-iodobenzyl alcohol [5], N-nitrosodipropylamine [8], butane-1,2,4-triol trinitrate [7, 10, 13] and [$^2H_5$,$^{15}N_3$]nitroglycerin [11, 12] have been used as internal standards.

The purpose of this paper is to describe a method using capillary GC with ECD for the quantitative determination of plasma levels of GTN with sufficient sensitivity, selectivity and reproducibility. Its limit of quantitation is 50 pg of GTN per ml of plasma. The method requires only 1 ml of plasma and a single extraction with hexane. The use of butane-1,2,4-triol trinitrate (BTN) as internal standard, chosen for its chemical similarity to GTN, allows for the losses of GTN during sample preparation. This technique appears practicable in all laboratories where capillary GC is more readily available than GC—MS.

EXPERIMENTAL

Chemicals and reagents

GTN was available as a 1% solution in ethanol (Merck 7753; Merck, Darmstadt, F.R.G.). The concentration of GTN was quantified as described by Suphajettra et al. [15].

The internal standard (BTN) was provided by the Société Nationale des Poudres et Explosifs (Vert-le-Petit, France) as a 1% solution in ethanol.

Nitrate metabolites of nitroglycerin (1,3- and 1,2-dinitroglycerols and 1- and 2-monoglycerols) were synthesized in our laboratories according to available methods [16].

Hexane [Pestipur, Solvants-Documentation-Synthèses (SDS), Peypin, France] is of analytical grade.

The aqueous solutions of nitroglycerin and internal standard are stored at 4° C and prepared freshly every month.

Equipment

A Hewlett-Packard Model 5880 A gas chromatograph equipped with computing integrator and electron-capture detector is used. The column is a 25 m × 0.32 mm fused-silica capillary column coated with methyl silicone (OV-1) (Hewlett-Packard 19091 A, Option 112). Splitless injection is used with an 18-sec splitless period. The column is flushed with the carrier gas (argon—methane, 90:10) at a flow-rate of 3 ml/min; the septum purge is 5 ml/min and the auxiliary gas flow to the detector 30 ml/min. The injector temperature is 150° C and the detector is set at 220° C. The column is at 40° C initially for 0.3 min and the temperature is then increased rapidly to 150° C for 5 min; then, to wash out plasma residues, it is raised rapidly to 250° C for 6 min.

The glassware is pretreated to prevent adsorption. It is immersed in toluene containing hexamethyldisilazane, trimethylchlorosilane and pyridine (1% each) for 15 min and rinsed with methanol. Then, it is washed for 30 min in an ultrasonic bath with methanol.

Extraction and gas chromatography

Of the internal standard solution 100 μl (5 ng of butane-1,2,4-triol trinitrate) are measured into a 10-ml glass centrifuge tube, to which 1 ml of plasma and 5 ml hexane are added. The tube is shaken mechanically (Infors shaker) for 20 min at 300 rpm and centrifuged at 2500 *g* for 2 min. An aliquot of the organic phase is transferred into a conical tube and concentrated to about 100 μl by evaporation under a nitrogen stream at 20°C (evaporation to dryness should be avoided).

A 1-μl portion of the hexane phase is injected into the gas chromatograph using the splitless injection technique.

Calibration curves

Calibration samples are prepared by introducing 100 μl of a suitable GTN aqueous solution (100—5000 pg) into 10-ml glass centrifuge tubes containing 1 ml of plasma and a constant amount of internal standard (5 ng). The calibration curve is obtained from the ln—ln plot of the peak height ratios versus the plasma concentrations. The equation is calculated as the ln—ln regression after subtraction of the blank determined during the calculation.

The samples for the calibration curve are prepared every week and stored at 4°C.

RESULTS AND DISCUSSION

Precision of calibration curves

The day-to-day precision of the standard curves was demonstrated by five consecutive experiments carried out on separate days. A ln—ln regression line with estimated blank value was generated from the 23 data points of the five standard curves (Table I). It corresponds to the equation $\ln (y + 0.010243) = 1.222 \ln x - 9.585$. A test of day-to-day precision was made by expressing each data point as a percentage of the value read off the ln—ln line for the corresponding concentration (Table I). The distribution of these normalized (concentration-independent) data had an average (± S.D.) of 101.7 ± 10.6%, demonstrating a good precision between experiments. However, a variability for the lower concentrations is noted which is why the samples for the calibration curve were injected daily and prepared weekly.

Within-day precision

The within-day precision of the method was checked by determining six plasma samples spiked with different concentrations of GTN. The results obtained with the procedure described above are given in Table II.

Day-to-day precision

The day-to-day precision was checked in plasma by determining four con-

TABLE I

DAY-TO-DAY PRECISION OF CALIBRATION CURVES USED TO DETERMINE NITROGLYCERIN IN PLASMA

Concentration added to plasma (pg/ml)	Peak height ratio						$100 \times \frac{E}{C}$
	Experimental (E)					Calculated from ln—ln with blank estimation regression line (C)	
	Day 1	Day 2	Day 3	Day 8	Day 9		
5000	2.275						100
		2.217					98
			2.587			2.267	114
				2.178			96
					2.113		93
2500	1.049						109
		1.026					106
			1.058			0.966	110
				0.932			96
					0.915		95
1000	0.319						104
		0.335					109
			0.314			0.308	102
				0.290			94
					0.277		90
250	0.046						96
		0.056					117
			0.042			0.048	88
				0.042			88
					0.041		85
100	—						—
		0.0110					124
			—			0.0089	—
				0.0095			107
					0.0105		118
Average ± S.D.							101.7 ± 10.6

centrations (250, 500, 2000 and 4000 pg/ml) of GTN, in duplicate, every day for one week. The results obtained with the procedure described are given in Table III.

Limit of quantitation

The precision was checked by determining six plasma samples spiked with 104 pg/ml GTN. The accuracy ranged from 90% to 110% with a mean value of 101%. The coefficient of variation for six determinations was 6.7% (Table IV).

Concentrations down to 52 pg/ml can be estimated with satisfactory precision but lower accuracy (Table IV).

TABLE II

WITHIN-DAY PRECISION OF THE ASSAY AND ACCURACY OF NITROGLYCERIN IN SPIKED PLASMA SAMPLES

	Amount introduced (pg/ml)*							
	250		500		2000		4000	
	Found (pg/ml)	Accuracy (%)	Found (pg/ml)	Accuracy (%)	Found (pg/ml)	Accuracy (%)	Found (pg/ml)	Accuracy (%)
	248	99	451	90	2095	105	3813	95
	255	102	441	88	2100	105	3875	97
	213	85	464	93	2077	104	3819	95
	252	101	448	90	2310	115	3774	94
	217	87	496	99	2102	105	3998	100
	220	88	486	97	1937	97	4522	113
Mean	234	94	464	93	2104	105	3967	99
Coefficient of variation (%)	8.3		4.8		5.7		7.1	
Mean accuracy ± S.D.			97.7 ± 7.8					

*1 pg/ml is equivalent to 4.405 pmol/l.

TABLE III

DAY-TO-DAY PRECISION AND ACCURACY OF NITROGLYCERIN IN SPIKED PLASMA SAMPLES

Day of analysis	Amount introduced (pg/ml)*							
	250		500		2000		4000	
	Found (pg/ml)	Accuracy (%)	Found (pg/ml)	Accuracy (%)	Found (pg/ml)	Accuracy (%)	Found (pg/ml)	Accuracy (%)
1	246	98	515	103	2088	104	3680	92
	289	116	503	101	2092	105	3875	97
2	261	104	460	92	2136	107	4039	101
	233	93	442	88	2294	115	4147	104
3	227	91	456	91	2034	102	4287	107
	189	76	463	93	1927	96	4447	111
4	269	108	499	100	2097	105	4096	102
	243	97	573	115	2047	102	3935	98
5	227	91	490	98	2035	102	4024	101
	273	109	490	98	2105	105	4361	109
Mean	246	98	489	98	2086	104	4089	102
Coefficient of variation (%)	11.7		7.7		4.5		5.7	
Mean accuracy ± S.D.			100.7 ± 8.0					

*1 pg/ml is equivalent to 4.405 pmol/l.

Plasma interference

Fig. 1 shows chromatograms of an extract of human plasma (1 ml) and of the same extract spiked with 2.8 ng of GTN and 5 ng of internal standard. No interference from normal plasma components was recorded.

TABLE IV

LIMIT OF QUANTITATION OF THE ASSAY OF NITROGLYCERIN IN SPIKED PLASMA SAMPLES

	Amount introduced (pg/ml)*			
	52		104	
	Amount found (pg/ml)	Accuracy (%)	Amount found (pg/ml)	Accuracy (%)
	63	121	101	97
	62	119	94	90
	57	110	107	103
	55	106	110	106
	61	117	114	110
	60	115	104	100
Mean	60	115	105	101
S.D.		5.7		7.0
Coefficient of variation (%)	5.2		6.7	

*1 pg/ml is equivalent to 4.405 pmol/l.

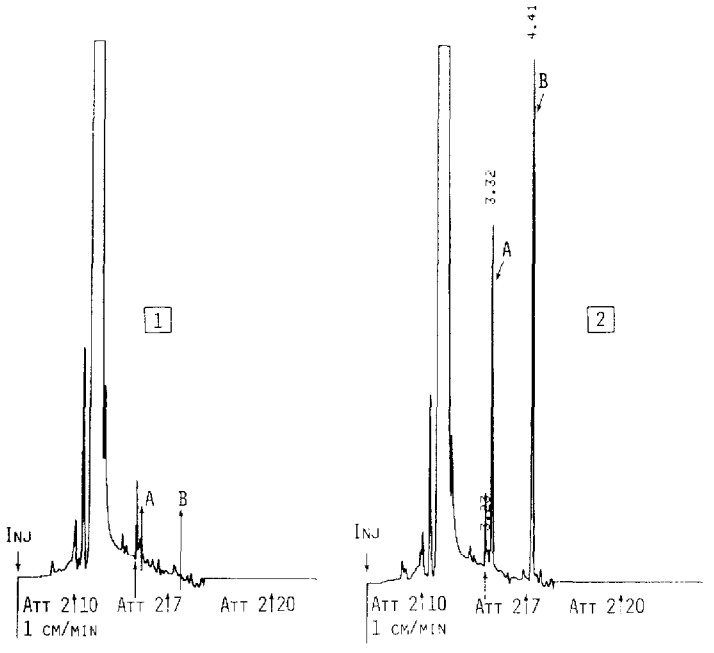

Fig. 1. Examples of chromatograms: (1) human plasma blank (1 ml of plasma); (2) same plasma spiked with 2.8 ng of nitroglycerin (A), and 5 ng of internal standard (B).

Hexane as extractive solvent

In the method described above, hexane (Pestipur, SDS) was used. It is obtained from a mixture of 50% paraffin C_6 *n*-hexane, 40% isoparaffin C_6

hexane, 10% cycloparaffin C_6 hexane, 100 ppm benzene, and 1 ppm sulphur. This hexane is distilled by the manufacturer as follows: from 100 l, the first 10% is discarded, 40% is distilled between 64°C and 68°C and the remainder is thrown away. The hexane used contains 95% of *n*-hexane and 5% of 3-methylpentane.

When *n*-hexane from Baker (reference 8205) with a boiling range 68.6—68.7°C was used, the extraction recovery was 7.5 times less than with hexane Pestipur, SDS, probably because it is pure *n*-hexane and not a mixture of isomers. This may explain why Yap et al. [2] had to extract GTN twelve times from plasma with equal volumes of *n*-hexane to obtain a good recovery (they used *n*-hexane from Fisher, which is probably pure *n*-hexane).

Selectivity

Nitrate metabolites of GTN (1,2- and 1,3-glyceryl dinitrates, 1- and 2-glyceryl mononitrates) were injected under the same conditions as GTN. These compounds were detected with retention times of 2.36 and 2.49 min for the mononitrates, and 3.0 and 3.06 min for the dinitrates. The retention time of GTN is 3.33 min, and it is clearly separated from its di- and mononitrate metabolites. Fig. 2 shows a chromatogram of a mixture of GTN and its four nitrate metabolites. Their simultaneous determination was not possible owing to their different polarities.

Comparison of the GC and GC—MS methods after analysis of control samples

Twelve spiked plasma samples with concentrations of GTN unknown to the analyst were determined using this GC method and the GC—MS method recently developed in our laboratories [14].

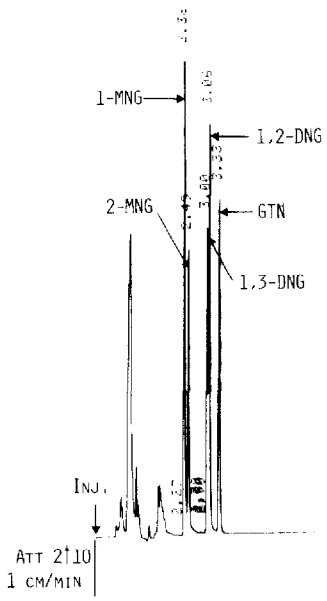

Fig. 2. Chromatogram of a mixture of 1- and 2-glyceryl mononitrate (MNG), 1,2- and 1,3-glyceryl dinitrate (DNG) and glyceryl trinitrate (GTN, nitroglycerin).

TABLE V

COMPARISON OF THE GC AND GC—MS METHODS FOR THE ASSAY OF NITROGLYCERIN IN SPIKED PLASMA SAMPLES ANALYSED IN A BLIND STUDY

Amount added (pg/ml)	GC—MS		GC	
	Found (pg/ml)	Accuracy (%)	Found (pg/ml)	Accuracy (%)
416	406	97.6	462	111.0
130	126	96.9	127	97.7
169	165	97.6	192	113.6
83	84	101.2	88	106.0
312	301	96.5	344	110.3
2080	2032	97.7	1937	93.1
936	912	97.4	914	97.6
208	205	98.6	211	101.4
2340	2246	95.9	2202	94.1
728	719	98.8	694	95.3
1560	1535	98.4	1565	100.3
1300	1271	97.8	1228	94.5
Mean accuracy ± S.D.		97.9 ± 1.3		101.2 ± 7.2

TABLE VI

COMPARISON OF THE GC AND GC—MS METHODS FOR THE ASSAY OF NITROGLYCERIN IN ACTUAL PLASMA SAMPLES (MEAN OF DUPLICATE ANALYSES)

Sample No.	Capillary GC (pg per 200 μl)	GC—MS (pg per 200 μl)	Percentage difference
8	1155	1121	3
9	1271	1378	8
10	599	640	6.5
11	309	304	1.5
12	222	197	12

The results obtained with the two procedures are given in Table V. The accuracy demonstrated by calculating the recovery for each method is 97.9 ± 1.3% and 101.2 ± 7.2%, respectively, for the GC—MS and GC methods.

Actual samples from a clinical pharmacology study with Trandermol-Therapeutic-System nitroderm in patients were analysed by the GC—MS [14] and the capillary GC methods. The results are in good agreement (Table VI). Fig. 3 shows the chromatogram of the actual sample 12.

The satisfactory agreement between the GC and GC—MS methods for the assay of GTN indicates that data obtained by both techniques give comparable results.

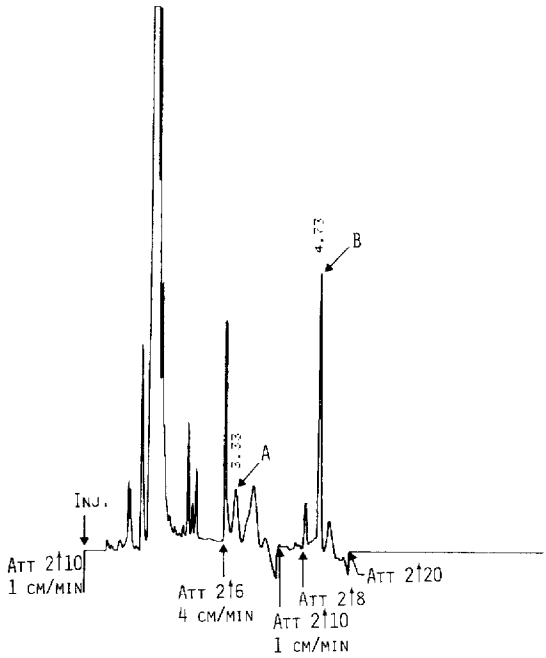

Fig. 3. Chromatogram of an actual plasma extract (200 μl) containing 222 pg of nitroglycerin (A) and 2.5 ng of internal standard (B).

CONCLUSION

This paper describes a sensitive, selective and precise capillary GC technique for the quantitative determination of GTN in human plasma at concentrations down to 50 pg/ml.

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