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# Note

## Simultaneous determination of glyceryl trinitrate and its two dinitrate metabolites in plasma and tissues by capillary gas chromatography

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Glyceryl trinitrate (GTN) is a potent vasodilator and is widely used in the treatment of angina pectoris. It is rapidly metabolized [1] to its active dinitrate metabolites, glyceryl 1,2-dinitrate (1,2-GDN) and glyceryl 1,3-dinitrate (1,3-GDN) [2]. As GTN and GDN are active at very low levels, a sensitive analytical method is required for their determination in plasma and tissues. Thin-layer chromatography (TLC) has been used for quantitation of labelled organic nitrates [3]. High-performance liquid chromatography (HPLC) has been developed for detection of GTN and its metabolites but the sensitivity, even with thermal energy analyzer detection, is inadequate for biological systems [4]. Several analytical methods based on gas chromatography (GC) have been developed to determine low concentrations of GTN [5–10] and the dinitrate metabolites [10,11] in plasma but only a few for the simultaneous quantitation of GTN and GDN [12–14]. However, these methods have disadvantages such as being time-consuming and difficult to automatize. In a previous animal study we found high levels of GTN in tissues such as brain, heart, aortic tissue and adipose tissue [15]. To our knowledge, the GDN levels in tissues have not been studied and no method for the determination of GDN has been published.

The aim of this work was to develop an uncomplicated and fast method for the simultaneous determination of GTN and its dinitrate metabolites in plasma *and* in tissues.

## EXPERIMENTAL

### *Chemicals and reagents*

GTN solutions were prepared from a 10% stock solution in ethanol (diluendum glyceryli nitratis 10%, Pharm Nord, Apoteksbolaget, Gothenburg, Swe-

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den). Dinitrate metabolites of GTN (1,2-GDN and 1,3-GDN) were provided by Dumex (Copenhagen, Denmark) as a 10% stock solution (>99%). The internal standard used was 99% *o*-dinitrobenzene (Aldrich-Europe, Beerse, Belgium). Ethanol was 99.5% (Kemetyl, Stockholm, Sweden). Pentane, dichloromethane and ethyl acetate (HPLC grade) were obtained from Lab-Scan Analytical Sciences (Dublin, Ireland).

*Equipment*

A Hewlett-Packard gas chromatograph 5890A (Palo Alto, CA, U.S.A.) equipped with a Hewlett-Packard 19233A $^{63}$Ni electron-capture detector, a Hewlett-Packard 7673A automatic sampler and a Hewlett-Packard 3393A computing integrator were used. The column was an Ultra-1 25 m × 0.32 mm I.D., 0.52 μm film thickness, cross-linked methylsilicone capillary column from Hewlett-Packard. Carrier gas was helium with a column flow-rate of 2.6 ml/min and a split of 1:6.8. Nitrogen at a flow-rate of 65 ml/min was used as the make-up gas. Splitless injection was used with a splitless period of 1 min. The injector temperature was 150°C and the detector was set at 250°C. The column temperature programme was set at 110°C initially and then programmed at 5°C/min to 124°C for 3 min, then at 10°C/min to 140°C and finally at 30°C/min to 165°C.

The liner used was linear for splitless injection, HP 18740-80220. The liner was silanized with a 20% (v/v) dimethyl dichlorosilane solution for 20 min and immediately rinsed with toluene and methanol before being dried at a temperature of 110°C. A glass wool wad was inserted into the liner and silanized on site with five 5-μl volumes of hexamethyldisilazane at 150°C.

*Extraction and gas chromatography*

Plasma (1 ml) was introduced onto an Extrelut 1 extraction column (Merck, Darmstadt, F.R.G.) and immediately eluted, without washing, with two 3-ml volumes of dichloromethane–pentane (1:1, v/v). The elute was evaporated with nitrogen at room temperature and the residue was reconstituted in 100 μl of internal standard solution, containing 24 ng/ml *o*-dinitrobenzene in ethyl acetate. A 2-μl aliquot was injected into the gas chromatograph. The higher boiling point of ethyl acetate compared to pentane was essential to maintain chromatographic precision, and no interference from the ethyl acetate was recorded.

Tissue was homogenized in two 3-ml volumes of dichloromethane–pentane (1:1) in a glass–glass homogenizer. The homogenate was transferred to tubes and centrifuged at 2000 *g* for 10 min at 5°C. The organic layer was removed and introduced onto a Extrelut 1 extraction column containing 1 ml of water. The column was then washed with 2 ml of dichloromethane–pentane (1:1). The eluate was evaporated with nitrogen at room temperature and the residue was reconstituted in 100–1000 μl of internal standard solution. Thereafter it was treated in the same way as the plasma samples.

*Standard curves*

Standard solutions containing GTN in ethanol were prepared fresh each day from the stock solution, and 1,2-GDN and 1,3-GDN in ethanol were prepared fresh every week from the stock solution. Standard samples were prepared in plasma. Concentrations for the low standard curve of GTN ranged from 0.1 to 5.0 ng/ml, while concentrations of 1,2-GDN and 1,3-GDN ranged from 0.5 to 10 ng/ml The high standard curve concentrations ranged from 5.0 to 40.0 ng/ml for GTN and from 10 to 100 ng/ml for 1,2-GDN and 1,3-GDN. Calibration curves were obtained by plotting peak-height ratios between the nitrates and the internal standard *versus* plasma concentrations of each of the three compounds.

## RESULTS AND DISCUSSION

*Instrumentation*

In recent studies it has been reported that GTN and also its metabolites may be adsorbed to active surfaces of capillary injectors, glass columns and detectors [8,14]. This is in accordance with our study, and therefore we used a silanized liner with silanized glass wool. The position of the column exit inside the detector were optimized for sensitivity, according to Noonan *et al.* [8]. Several fused-silica capillary columns coated with methyl silicone or phenylmethyl silicone were tested during the development of this assay. The HP-5 column from Hewlett-Packard and DB-5 (Durabond-5) column from J&W Scientific (Rancho Cordova, CA, U.S.A.) had either poor resolution or poor detectability. The PTE-5 column from Supelco (Bellefonte, PA, U.S.A.) exhibited poor separation for the two dinitrates. The Ultra-1 column was choosen for the assay due to its good separation characteristics and high detectability (Fig. 1). The retention times for the components

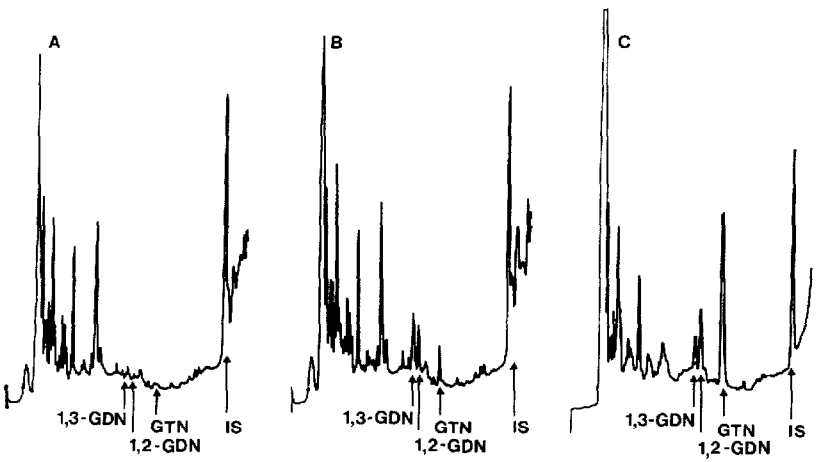


Fig 1 Gas chromatograms of (A) blank plasma, (B) plasma sample spiked with 0 5 ng/ml GTN and 1.0 ng/ml 1,2-GDN and 1,3-GDN and 24 ng/ml *o*-dinitrobenzene (internal standard, IS) and (C) tissue extract from adipose tissue containing 456 ng/g GTN, 194 ng/g 1,2-GDN and 110 ng/g 1,3-GDN.

were 4.28 min for 1,3-GDN, 4.47 min for 1,2-GDN, 5.23 min for GTN and 7.63 min for *o*-dinitrobenzene.

*Extraction and recovery*

Pentane as extraction solvent resulted in a 100% recovery of GTN from plasma [15], but for GDN it showed an extremely poor recovery. Diethyl ether, dichloromethane, chloroform and pentane–ethyl acetate (1:9) were tested as extraction solvents, but they all showed a recovery of less than 50%. Further development resulted in a mixture of dichloromethane–pentane (1:1), a mixture that Lee *et al.* [14] had studied by liquid–liquid extraction. It is also known that when a conventional liquid–liquid extraction method is shifted to column extraction, a significantly better recovery is often reached. By using Extrelut extraction columns we were able to increase the recovery. The overall recovery was not estimated since, for unknown reasons, the peak heights obtained of the 100% solution in ethyl acetate was lower than those of the extracted ones, a phenomenon recognized on every occasion studied. The extraction recoveries were estimated from 100% solutions of GTN, 1,2-GDN and 1,3-GDN made in dichloromethane–pentane (1:1) and then evaporated with nitrogen (Table I). It is unclear why the concentration in the 100% solution of GTN and GDN in ethyl acetate is lower than the extracted solution, but degradation of the substances, when diluted directly from ethanol to pure ethyl acetate, could be an explanation. The evaporation of the extraction solvents has been a critical step in some methods and it has not been possible to evaporate to complete dryness [12]. Probably, the choice of extraction solvent in this method conduced to negligible losses of either substances at the evaporation step.

*Precision studies*

The overall precision of this method was checked by analysis of nine plasma

TABLE I

EXTRACTION RECOVERY OF GTN, 1,2-GDN AND 1,3-GDN FROM PLASMA

| Compound | Mean recovery ($n=2$) (%) | |
|---|---|---|
| | Low concentration (0.1 ng/ml GTN) (0.5 ng/ml GDN) | High concentration (40 ng/ml GTN) (100 ng/ml GDN) |
| GTN | 84 | 117[a] |
| 1,2-GDN | 68 | 85 |
| 1,3-GDN | 108 | 87 |

[a] See comment in Results and discussion section

TABLE II

OVERALL PRECISION FOR GTN, 1,2-GDN AND 1,3-GDN IN PLASMA

| Concentration (ng/ml) | Coefficient of variation ($n=9$) (%) | | |
|---|---|---|---|
| | 1,2-GDN | 1,3-GDN | GTN |
| 0.1 | | | 9 6 |
| 0 5 | 9.6 | 5 4 | |
| 5 0 | | | 8 6 |
| 10 0 | 6.4 | 6 5 | |
| 40 0 | | | 4 6 |
| 100.0 | 3.2 | 3 5 | |

samples taken from a pool, to which known amounts of GTN, 1,2-GDN and 1,3-GDN were added. The coefficients of variation are listed in Table II.

Non-linear standard curves of GTN and GDN have been reported over a large concentration range [12,14]. This is in accordance with our results, and therefore two linear standard curves were used for GTN (0.1–5.0 and 5.0–40 ng/ml) and for GDN (0.5–10 and 10–100 ng/ml). The curves were linear in the low concentration range up to 5.0 and 10 ng/ml for GTN and GDN, respectively ($r=0.9966$ for 1,3-GDN; $r=0.9989$ for 1,2-GDN; $r=0.9969$ for GTN). The calibration curves were linear in the high concentration range up to 40 and 100 ng/ml for GTN and GDN respectively ($r=0.9990$ for 1,3-GDN; $r=0.9989$ for 1,2-GDN, $r=0.9997$ for GTN).

*GTN contamination*

It is well known that GTN is absorbed to plastic materials [16,17], and as mentioned above GTN also adsorbs to glassware. During development work of the low concentration range, we noticed a cross-contamination of GTN in plasma samples. If a pipette with exchangeable tips (Eppendorf Varipette 100–1000 $\mu$l) was used both for the stock solution of GTN (0.44 *M* in ethanol) and for the blank plasma, the blanks were found to contain 0.1–1.0 ng/ml GTN, which is in the range of our low concentration curve. When using the pipette for subsequent blanks the amount of GTN was gradually decreased. This complication of contamination by GTN vapor should not be underestimated, and to our knowledge this has not been previously reported.

## CONCLUSION

The present method for simultaneous quantitation of GTN and its dinitrate metabolites in human plasma and tissue homogenates involves only one single

step of extraction. The single extraction, the automatic sampler and the short chromatographic time gives the method advantages compared to other methods. It is an uncomplicated and fast method, with a capacity for a large amount of samples. The described method is the first one for the simultaneous quantitation of GTN, 1,2-GDN and 1,3-GDN in plasma and tissue such as brain, heart, aortic and adipose tissue.

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