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Note

Determination of nitroglycerin in human plasma using bonded-phase capillary column gas chromatography with electron-capture detection

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Nitroglycerin (1,2,3-propanetriol trinitrate; TNG) is a potent vasodilator. As it is active probably even at levels as low as 0.1 ng/ml in plasma [1], sensitive analytical methods are required for its determination in plasma. Moreover, TNG is rapidly metabolized [2,3], yielding lower nitrate esters of glycerin, viz. propanetriol 1,2-dinitrate (1,2-DNG), propanetriol 1,3-dinitrate (1,3-DNG), propanetriol 1-nitrate (1-MNG) and propanetriol 2-nitrate (2-MNG). All of the lower nitrated esters of glycerin possess significant biological activity, similar to that of TNG [4].

Although various methods have been described for the detection of either TNG or its metabolites in human plasma (for a review see, e.g., ref. 1 and more recent papers [5–7]), none of the published methods was suitable for the simultaneous determination of both TNG and its metabolites. The plasma levels of TNG after oral administration of sustained-release TNG dosage forms were reported by Blagodarskaya [8] and Noonan and Benet [5] to be of the order of 0.1–0.2 ng/ml. The methods applied were packed-column gas chromatography with electron-

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capture detection (GC–ECD) [8] or capillary gas chromatography–mass spectrometry (GC–MS) [5].

Capillary GC–ECD proved to be an effective final step for the determination of either TNG or its metabolites in plasma extracts. However, no suitable extraction system for both TNG and the lower nitrates has been published. Hydrocarbons (e.g., pentane, hexane) do not extract the polar lower nitrates from plasma [4–7] and more polar solvents such as ethyl acetate and dichloromethane [10,11] yield very impure extracts that are not suitable for the GC–ECD determination of TNG.

The aim of this work was to develop a method for the simultaneous determination of both TNG and its metabolites in human plasma after oral administration of sustained-release TNG dosage forms.

EXPERIMENTAL

Reagents

A ca. 2% ethanolic solution of TNG (VCHZ, Pardubice, Czechoslovakia) was used to prepare more dilute TNG solutions. The exact concentration of the 2% stock solution was checked by titrimetry [9]. 2-MNG and 1,2-DNG in addition to MNG and DNG mixtures enriched with 1-MNG and 1,3-DNG, respectively, were prepared at the Drug Research Institute (Modra, Czechoslovakia) and were stored as ca. 2% ethanolic solutions. Their purity was checked by GC and the exact concentration was checked by automated nitrogen analysis (Model 1106 CHN analyser, Carlo Erba, Milan, Italy). 1,3-Dinitrobenzene, *n*-pentane (Uvasol grade) and ethyl acetate (Uvasol grade) were obtained from E. Merck (Darmstadt, F.R.G.) and were used as received. *n*-Hexane (analytical-reagent grade, Fluka, Buchs, Switzerland) was purified by column distillation from all-glass apparatus. Other chemicals were analytical-reagent grade products from Lachema (Brno, Czechoslovakia) and were used as received.

Chromatographic system

A Hewlett-Packard HP-5880A gas chromatograph equipped with a universal injection port (HP 19320A) and a single ^{63}Ni electron-capture detector (HP 19303) was used. An HP-1 (Hewlett-Packard) cross-linked methylsilicone-coated fused-silica wide-bore capillary column (10 m × 0.53 mm I.D., 2.65 μm film thickness) was used. The injector was operated in the splitless mode at 100 °C and the detector was kept at 220 °C. The column temperature was initially held at 50 °C for 2 min, then programmed at 30 °C/min to 80 °C and then at 5 °C/min to 150 °C, the final temperature being held for 1 min. After the run, the plasma residues were baked out of the column at 180 °C for 10 min.

Sample preparation

Silanized glassware was used throughout the sample preparation. Samples of 4 ml of blood were collected in chilled heparinized Pyrex test-tubes containing 0.1 ml of 1.0 mol/l silver nitrate solution. The blood was immediately centrifuged (2000 *g*) in a refrigerated centrifuge at 4 °C and 2 ml of plasma were transferred

into another test-tube. A 50-μl volume of internal standard solution (a 0.114 μg/ml ethanolic solution of 1,3-dinitrobenzene) was added and the sample was extracted with 2 ml of extraction solvent (*n*-pentane–ethyl acetate, 1:9). A 1-ml volume of the organic phase was evaporated to dryness at laboratory temperature in a dry conical test-tube under a gentle stream of nitrogen. The residue was dissolved in 30 μl of *n*-heptane and a 0.5-μl aliquot was immediately injected into the GC system.

Data evaluation

The compounds of interest were quantitated by the internal standard method based on the peak-area ratio. It was assumed that 1-MNG and 1,3-DNG exhibit the same response as 2-MNG and 1,2-DNG, respectively. A standard least-squares procedure was used to evaluate the parameters of the calibration graph.

RESULTS AND DISCUSSION

The method was found to be reproducible and very sensitive, with a detection limit in the sub-picogram range for direct injection of TNG.

The extraction recovery for all the compounds studied was satisfactory even though a single extraction was used. The recoveries at two levels of analyte concentration are summarized in Table I.

Chromatograms obtained with both blank and spiked extracts are shown in Figs. 1 and 2. Even though both chromatograms show numerous plasma peaks, none of them interfered either with the compounds of interest or with the internal standard. All the peaks were clearly integrated by standard integrator software.

The calibration graphs were linear over the range tested. The calibration points for TNG covered the range 100–1200 pg/ml (five points), for DNG 50–500 pg/ml and for MNG 200–1500 pg/ml (four points in each instance). In contrast to previous findings [5,11], we experienced no problems with long-term reproducibility. The long-term reproducibility of the assay technique is exemplified in Table II, using five random sampling points spread over a four-month bioavailability study.

The detection limit for the compounds of interest was not limited by the final GC–ECD step but rather by the blank values. Owing to the high volatility of TNG

TABLE I

EXTRACTION RECOVERY OF THE PROPOSED PROCEDURE

Compound	Mean recovery ($n=5$) (%)	
	200 pg/ml added	500 pg/ml added
TNG	103 ± 5	86 ± 6
1,2-DNG	70 ± 10*	67 ± 8
2-MNG	99 ± 4	75 ± 9

*100 pg/ml added.

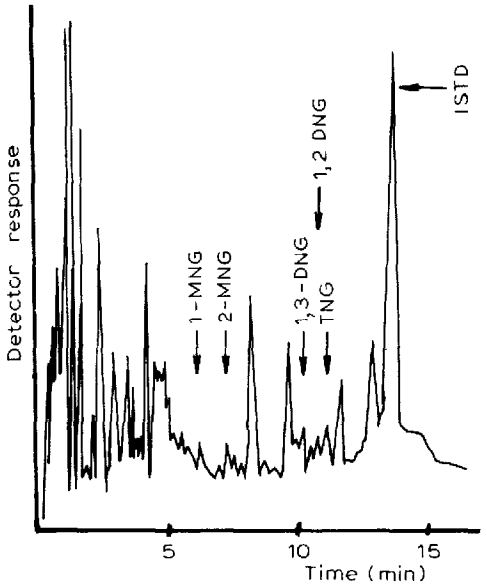

Fig. 1. Chromatogram of blank plasma extract. The small but clearly detectable peaks of TNG and 1-MNG are due to the ubiquity of these compounds.

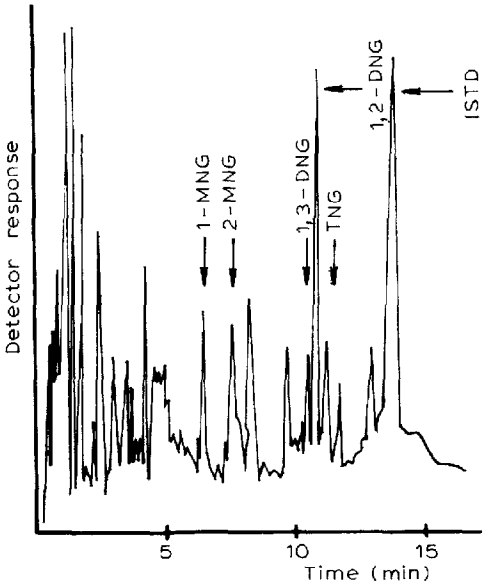

Fig. 2. Chromatogram of plasma sample spiked at 500 pg/ml with 1,2-DNG and with 100 pg/ml of the other four compounds.

(and also of its metabolites) these compounds are claimed to be ubiquitous [11] and once the sensitivity of the analytical method reaches a certain value they are found in any material used in the laboratory. Using modest quarantine precautions we were able to reduce the blank TNG levels to about 40 pg/ml and to about 50 pg/ml for both DNG and MNG. These figures also represent the operational limit of detection for the respective compounds.

TABLE II

LONG-TERM REPRODUCIBILITY OVER FOUR MONTHS

Data were all calculated using a single calibration graph.

Compound	Amount found (mean ± S.D., $n=5$) (pg/ml)	
	200 pg/ml added	500 pg/ml added
TNG	189 ± 33	466 ± 37
1,2-DNG	88 ± 8*	404 ± 66
2-MNG	201 ± 15	448 ± 72

*100 pg/ml added.

CONCLUSIONS

The feasibility of using *n*-pentane–ethyl acetate extraction for the simultaneous GC–ECD determination of TNG and its metabolites in human plasma at the sub-nanogram per millilitre levels has been demonstrated. The simple and effective extraction procedure together with the sensitivity, reproducibility and reliability of capillary GC–ECD with an inert and rugged fused-silica bonded-phase wide-bore capillary column have resulted in a reproducible and relatively simple to perform analytical procedure. The method has been used for the quantitation of TNG and its metabolites in a bioavailability study, involving the administration of three sustained-release TNG preparations over about four months.

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