Improved GLC Determination of Plasma Nitroglycerin Concentrations

PETER S. K. YAP, EDWARD F. McNIFF, and HO-LEUNG FUNG ˣ

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Abstract ☐ A specific and quantitative assay for determining nanogram levels of nitroglycerin in plasma was developed. The method involves stabilization of the drug in plasma with silver nitrate, followed by multiple extraction using purified hexane. Isosorbide dinitrate is added as the internal standard. The hexane extract is subsequently concentrated and injected into a glass column packed with 3% SP-2401 on 100–120-mesh Supelcoport at 140°. A ^{63}Ni-electron-capture detector gives a linear response over the range of 0.1–50 ng of nitroglycerin/ml of rat plasma. From spiked samples, the procedure gave a recovery of about 90%. There was little or no interference from the isomeric glyceryl dinitrates and endogenous compounds in rat or human plasma.

Keyphrases ☐ Nitroglycerin—GLC analysis in plasma ☐ GLC—analysis, nitroglycerin in plasma ☐ Vasodilators, coronary—nitroglycerin, GLC analysis in plasma

Rosseel and Bogaert (1, 2) recently reported a GLC procedure for the determination of nitroglycerin in human plasma with a claimed sensitivity of 0.5 ng/ml of plasma. That procedure represents a major advance in the analysis of trace levels of organic nitrates in biological fluids, but several problems are associated with it. First, to reach the claimed sensitivity, a volume of 5 ml of plasma/sample must be processed. This sample size severely limits the utilization of the assay in pharmacokinetic studies of nitroglycerin in small animals, *e.g.*, rats, from which serial withdrawal of large volumes of blood is impossible. Second, the chromatogram contains major interference peaks close to those of nitroglycerin and the internal standard, isosorbide dinitrate, making drug quantitation at lower concentrations much less reliable. Third, a total chromatogram time of 90 min is required because of the presence of some large peaks due to solvent impurities. This relatively long assay time impairs the efficiency of the procedure.

The present report describes attempts to develop a better assay for nitroglycerin in plasma. Modifications of the Rosseel and Bogaert assay were developed and appeared to remedy successfully the shortcomings mentioned.

EXPERIMENTAL

Standard Solutions—Nitroglycerin stock solutions in hexane[1] were prepared by extraction of a commercial 10% (w/w) nitroglycerin–lactose adsorbate[2] with the solvent and standardized by a modified USP method in which column elution was omitted and isooctane was substituted by hexane as the extraction solvent. Aqueous nitroglycerin stock solutions were prepared by dissolving the nitroglycerin–lactose in distilled water and standardized by a modified USP method in which the nitroglycerin was adsorbed onto siliceous earth in a beaker rather than on a column. The modified procedures gave the same results as the official method.

Isosorbide dinitrate, the internal standard, was provided[3] as a 25% (w/w) isosorbide dinitrate–lactose powder. To obtain the lactose-free internal standard, an aqueous solution of the powder was extracted with ether, which was then evaporated to dryness. The resultant solid was recrystallized from an aqueous alcoholic mixture, mp 70–71° (uncorr.) [lit. (3) mp 70°].

Purification of Hexane—About 600 ml of reagent grade hexane was washed twice with 300 ml of concentrated sulfuric acid, twice with 300 ml of distilled water, once with 300 ml of 1 *N* NaOH, and then twice with 300 ml of distilled water. The washed solvent was then passed through a column of silica gel[4] (about 50 g, packed as a slurry into a column 2 cm in diameter and 30 cm in height) and distilled in the presence of activated charcoal and lithium aluminum hydride. Each batch of solvent was tested by concentrating 2.4 ml of the purified solvent to about 20 μl (~120-fold), and a volume of 1–5 μl was injected onto the gas chromatograph. The organic solvent was approved for use in the assay when interfering impurity peaks were absent.

Extraction and GLC Assay—A volume of 0.2 ml of purified hexane was used for each extraction of an equal volume of plasma containing nitroglycerin. Rapid injection of the organic solvent into the plasma provided the required mixing, with minimal formation of an emulsion. The hexane layer (upper phase) was withdrawn, and the extraction was repeated for the specified number of times. In this manner, 12 extractions could be accomplished easily within 5 min. The hexane extracts were combined, and a known amount of isosorbide dinitrate in hexane was added.

The internal standard was added after extraction because preliminary experiments indicated that isosorbide dinitrate was not completely extracted by the present procedure. The amount of internal standard added depended on the nitroglycerin level to be determined. For samples containing about 0.1 ng of nitroglycerin/ml, about 0.1 ng of isosorbide dinitrate in 10 μl of hexane was added to each sample. Larger amounts of internal standard were added for samples containing higher drug levels to optimize GLC readings.

The combined hexane solution containing nitroglycerin and isosorbide dinitrate was then concentrated to approximately 20 μl, and 1–5 μl was injected into a glass column[5] packed with 3% SP-2401 on 100–120-mesh Supelcoport at a carrier gas (nitrogen) flow rate of about 60 ml/min. Inlet, column, and detector oven temperatures were set at 160, 140, and 180°, respectively. Quantification was effected *via* a ^{63}Ni-electron-capture detector[6].

RESULTS AND DISCUSSION

Use of Purified Hexane as Extracting Solvent—Rosseel and Bogaert (1, 2) showed that nitroglycerin in plasma can be extracted totally into ethyl acetate. From a recovery standpoint, ethyl acetate is a good extracting solvent because it has a superior solubility for nitroglycerin compared to other water-immiscible solvents. However, from the analytical point of view, ethyl acetate is a poor solvent because of its relative polarity and the difficulty in ridding it of contaminants. The polar plasma constituents it apparently extracts and the impurities it contains not only interfere with the quantitation of nitroglycerin and its major metabolites in the chromatogram but also limit the possible sensitivity of detection.

In this situation, a hydrocarbon solvent such as hexane has the opposite characteristics when compared to ethyl acetate. It is relatively easy to

[1] Fisher Scientific Co., Chemical Manufacturing Division, Fair Lawn, NJ 07410.
[2] Nitroglycerin 10% (w/w) in lactose, lot B17-H-1, ICI America, Atlas Chemical Division, Wilmington, DE 19899.

[3] Stuart Pharmaceuticals, Division of ICI United States, Wilmington, DE 19897.
[4] Silica gel Woelm for dry-column chromatography, Waters Associates, Framingham, MA 01701.
[5] Supelco, Bellefonte, PA 16823.
[6] Packard model 884 electron-capture detector at 40 v and Series 7500 gas chromatograph, Packard Instrument Co., Downers Grove, IL 60515.

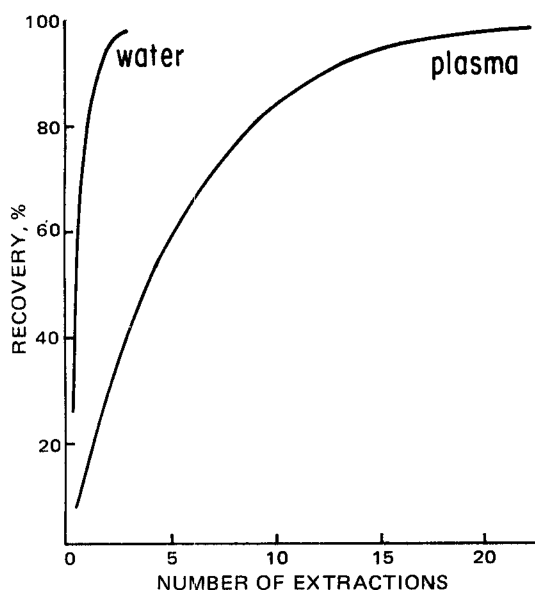

Figure 1—*Estimated percent nitroglycerin recovery from water and rat plasma* versus *number of extractions required. The curves are simulated using a phase volume ratio of 1 and partition coefficients of 4 and 0.2 for hexane–water and hexane–rat plasma, respectively.*

purify and does not extract as many polar plasma interfering substances. Its main disadvantage is, of course, its poor solubility for nitroglycerin. The partition coefficient of nitroglycerin between hexane and water was about 4 at room temperature. Between hexane and plasma, however, the partition coefficient was much lower, having a value of about 0.2. The much higher apparent solubility of nitroglycerin in plasma *versus* water may be attributed to plasma proteins and lipids that bind and/or solubilize the drug. Figure 1 shows the calculated (4) percent recovery of nitroglycerin as a function of the number of extractions from water and rat plasma, using an equal volume of hexane. For 90% recovery of nitroglycerin from rat plasma, 10 extractions would be required. Similarly, 21 extractions would enable a recovery of 99%.

Experimentally, a recovery of about 90% was achieved when nitroglycerin in spiked rat plasma was immediately extracted 12 times with equal volumes of hexane. When six extractions were performed, the recovery was 62%. These observed recovery percentages are consistent with the values predicted from the partition coefficient. The extraction efficiency of hexane from human plasma was similar to that from rat plasma. After 12 extractions, the nitroglycerin recovery was 92%.

In this assay, the combined hexane extract was concentrated to approximately 20 μl rather than to dryness followed by reconstitution as described in the procedure of Rosseel and Bogaert. This step prevented significant nitroglycerin losses (about 40%) when the hexane was totally evaporated. Loss of drug from total solvent evaporation was not prevented when ethyl acetate was added at 30% (v/v) to the combined hexane extracts. Complete recovery was obtained, however, when the hexane phase was concentrated to about 20 μl prior to injection onto the GLC column.

Stabilization of Nitroglycerin by Silver Nitrate—DiCarlo and Melgar (5) showed that nitroglycerin is rapidly degraded in rat serum; the half-life was approximately 20 min at 37°. Moreover, nitroglycerin degradation in rat serum can be inhibited significantly by iodoacetamide, silver nitrate, and *p*-chloromercuric benzoate (5). Silver nitrate was chosen for stabilization here because it is very poorly soluble in hexane and thus was not expected to cause additional problems during chromatography.

In fresh rat plasma, nitroglycerin degraded rapidly at ambient temperatures; approximately 25% of the original concentration was lost within 1 hr. Addition of silver nitrate to a final concentration of 0.05 *M* appeared to stabilize the drug for at least 1.5 hr, and only 20% of the nitroglycerin was destroyed after 12 hr. Addition of silver nitrate at the same final concentration to rat blood, however, resulted in extensive gelling of the sample, making plasma separation for drug analysis very difficult. Therefore, stability studies of nitroglycerin in whole blood spiked with silver nitrate were not conducted.

Adopted Procedure—Based on the previous findings, a final analytical procedure was adopted. For each 200-μl plasma sample, 10 μl of

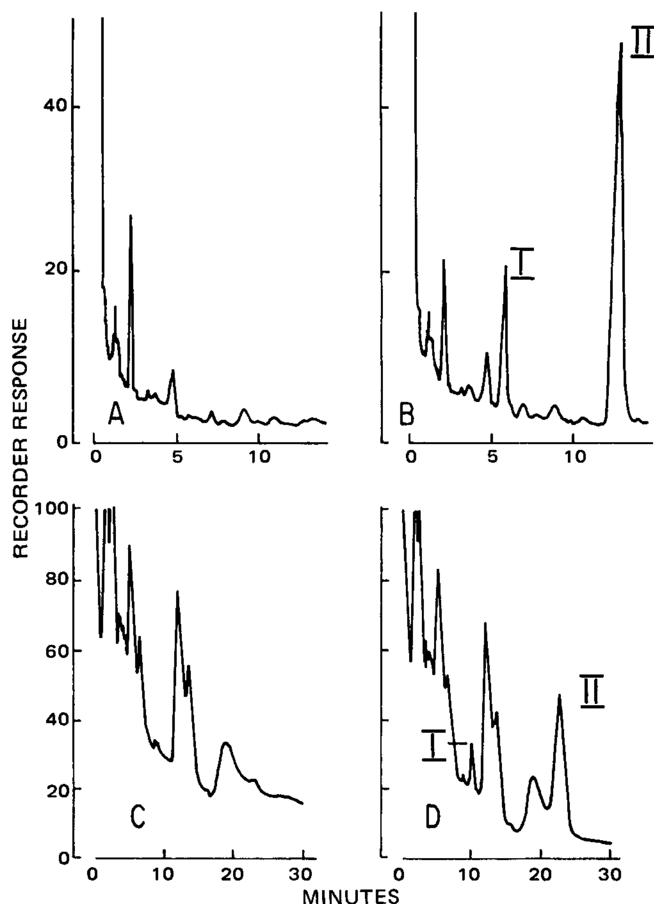

Figure 2—*Chromatograms of human plasma (A) and of human plasma with 4 ng of nitroglycerin/ml (B) as determined by the present procedure (electrometer setting of 8 × 10^{-10} amp), and chromatograms of human plasma (C) and of human plasma containing an estimated 3 ng of nitroglycerin/ml (D) as reported in Ref. 2 (electrometer setting of 1 × 10^{-9} amp). Key: I, nitroglycerin; and II, isosorbide dinitrate.*

1 *M* silver nitrate was first added; this sample was then extracted 12 times with equal volumes of purified hexane. After the internal standard was added to the combined extracts, the hexane solution was evaporated to approximately 20 μl under nitrogen; 1–5 μl of the concentrated solution was injected into the gas chromatographic column.

The present procedure offered a relatively "clean" chromatogram for rat and human plasma (Fig. 2A). Under the conditions used, the retention times of nitroglycerin and isosorbide dinitrate were 6 and 13 min, respectively. Occasionally, minor solvent impurity peaks were present, but they were broad and appeared between 25 and 35 min after injection. Figure 2B shows a chromatogram of human plasma spiked with 4 ng of nitroglycerin/ml. Chromatograms of human plasma (Fig. 2C) and human plasma estimated to contain about 3 ng of nitroglycerin/ml (Fig. 2D), as reported by Rosseel and Bogaert (2), are presented for comparison. The present procedure is superior when chromatogram clarity and assay time are considered.

The chromatographic peak of the isomeric dinitrates appeared at a retention time (about 3 min) at which there was no significant interference by other substances in the plasma and solvent. Although the extraction of dinitrates into hexane was poor (about 10–20%), the present nitroglycerin chromatogram offers a qualitative estimation of the dini-

Table I—Precision of GLC Assay of Nitroglycerin

Plasma Nitroglycerin Concentration, ng/ml		
Spiked	Observed (mean ± *SD*)	Range (*n*)[a]
0.095	0.099 ± 0.023	0.075–0.130 (7)
0.475	0.401 ± 0.095	0.291–0.504 (7)
2.00	1.95 ± 0.17	1.90–2.20 (6)
39.7	38.0 ± 1.6	35.1–40.0 (6)

[a] *n* = number of determinations.

trate level in the same plasma sample. This estimation apparently cannot be obtained directly from the Rosseel–Bogaert assay because of large interference peaks early in the chromatogram.

Linear calibration plots for nitroglycerin in rat and human plasma were obtained with the present procedure. The correlation coefficients of the lines were greater than 0.99. The precision and reproducibility of the present assay applied to plasma samples are shown in Table I. As expected, the relative standard deviations were higher ($\simeq$20%) for samples containing low concentrations of nitroglycerin (0.1 and 0.5 ng/ml) than for those with high concentrations. At 2 and about 40 ng/ml, the relative standard deviations were determined to be 8.6 and 4.2%, respectively. Saturation of the electron-capture detector occurred when the on-column amount of nitroglycerin exceeded about 0.5 ng.

Applications of the present technique in pharmacokinetic studies of nitroglycerin in a human subject (6) and in rats (7) are described elsewhere.

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Pharmacokinetics of Nitroglycerin in Rats

PETER S. K. YAP and HO-LEUNG FUNG ˣ

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Abstract □ The plasma nitroglycerin levels obtained after intracardial (0.7 mg/kg), oral (7 mg/kg), and topical (7–14 mg/kg) doses of nitroglycerin in rats are reported. Nitroglycerin followed essentially one-compartment kinetics after intracardial administration, showing a mean half-life of about 4 min and a mean apparent volume of distribution of about 3 liters/kg. After oral drug administration, "flip-flop" kinetics were evident. The mean oral bioavailability was determined to be 1.6%, firmly supporting the contention that nitroglycerin is extensively metabolized during first passage through the liver. Under the experimental conditions studied, no detectable levels of nitroglycerin were observed after topical application.

Keyphrases □ Nitroglycerin—pharmacokinetics after intracardial, oral, and topical administrations in rats □ Pharmacokinetics—nitroglycerin after intracardial, oral, and topical administrations in rats □ Vasodilators, coronary—nitroglycerin, pharmacokinetics after intracardial, oral, and topical administrations in rats

Recent studies dealt with the analytical (1–3), stability (4–7), and formulation (6, 8–12) aspects of nitroglycerin. These studies showed definitively that nitroglycerin in sublingual tablets can be stabilized effectively against volatilization by the inclusion of macromolecules such as povidone (8–10), polyethylene glycol (6), and microcrystalline cellulose (8, 12).

BACKGROUND

Very little information is available, however, on the pharmacokinetics of nitroglycerin in animals and humans. The analytical difficulty associated with the determination of nanogram or subnanogram concentrations of the intact drug in biological fluids is a primary reason for the paucity of quantitative information regarding nitroglycerin absorption and disposition. Recently, a GLC assay (13) quantifying nitroglycerin in plasma at these low concentrations was developed. This assay offers a unique opportunity to initiate rigorous *in vivo* pharmacokinetic studies in animals and humans.

Nitroglycerin is used in oral sustained-release dosage forms for the prophylaxis of angina. The effectiveness of this mode of nitroglycerin administration was seriously questioned by Needleman *et al.* (14) who showed that the drug undergoes extensive first-pass metabolism after oral dosing. The intact nitroglycerin levels were much lower after oral administration than after the intravenous route. Their data, however, were not reported in sufficient detail to allow for the estimation of the bioavailability of an oral dose of nitroglycerin.

Recently, nitroglycerin was used in ointment form for the hemodynamic management of patients with chronic congestive heart failure (15). This administration route for nitroglycerin was reported to produce beneficial effects lasting for 3–6 hr after a single application. The therapeutic efficacy and duration of effect of nitroglycerin, therefore, appear to be highly dependent on the route of drug administration.

The present study deals with the effect of the administration route on nitroglycerin pharmacokinetics in the rat. A pilot, collateral study in humans after therapeutic sublingual, oral, and topical doses of nitroglycerin is reported elsewhere (16).

EXPERIMENTAL

Materials—A 1.16-mg/ml aqueous solution of nitroglycerin was prepared by dissolving the appropriate amount of 10% nitroglycerin–lactose adsorbate[1] in distilled water. Solution was effected by overnight mechanical agitation and confirmed by the kinetic assay (2). The internal standard, isosorbide dinitrate, was obtained by acetone extraction from a 25% (w/w) isosorbide dinitrate–lactose powder[2], followed by evaporation of the organic solvent. Male Sprague–Dawley rats, 270–340 g, were used.

Intracardial Experiments—Six rats were fasted overnight and given an intracardial dose of nitroglycerin (0.7 mg/kg) under ether anesthesia. The dosing volume was approximately 0.2 ml. Blood samples (0.5–0.8 ml) were obtained through orbital sinus puncture using heparinized capillary tubes. Half of the rats (A, B, and C) were anesthetized during blood

[1] Nitroglycerin 10% (w/w) in lactose, ICI America, Atlas Chemical Division, Wilmington, DE 19899.

[2] Stuart Pharmaceuticals, Division of ICI United States, Wilmington, DE 19897.