Determination of Picogram Nitroglycerin Plasma Concentrations Using Capillary Gas Chromatography with On-Column Injection

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Abstract □ A specific, sensitive, and precise capillary gas chromatographic (GC) assay capable of analyzing picogram concentrations of nitroglycerin in human plasma was developed. The analytical procedure involves a double extraction of 1 mL of plasma with pentane, after the addition of internal standard (1 ng of 2,6-dinitrotoluene), followed by evaporation and reconstitution in 50 μL of heptane. The extract (1 μL) was injected onto a capillary column using the on-column injection technique. The GC oven temperature was programmed from 120°C to 180°C at a rate of 5°C/min. The oven temperature was then programmed to 250°C and was maintained for 10 min. The nitroglycerin and internal standard retention times were 8.6 and 11.4 min, respectively. The position of the end of the capillary column inside the detector is a critical determinant of sensitivity: the column exit must be positioned such that nitroglycerin adsorption to the detector is minimized (*i.e.*, sensitivity maximized). The assay limit of quantitation was 25 pg/mL (CV = 7.6%) using 1 mL of plasma. This GC assay, specific for nitroglycerin in the presence of its metabolites, isosorbide dinitrate, and several other drugs, may be used to quantitate plasma levels obtained after therapeutic nitroglycerin doses.

Keyphrases □ Nitroglycerin—GC, picogram quantitation □ GC—nitroglycerin, picogram quantitation

Nitroglycerin is a potent vasodilator used in the treatment of angina pectoris and congestive heart failure associated with acute myocardial infarction (1). The pain of angina may be relieved and/or prevented by the administration of sublingual, oral, or topical nitroglycerin. Although a definitive therapeutic plasma concentration range has not yet been defined, it is still important to identify the nitroglycerin concentration–response relationship so that dose adjustments in patients may be made more effectively. Nitroglycerin administration results in venous plasma concentrations ranging from <50 pg/mL to 10 ng/mL, depending on the route of administration (2–4). Studies in the rhesus monkey (5) indicate that concentrations of ^{14}C-labeled nitroglycerin metabolites are 10- to 300-fold higher than the parent drug. Therefore, an analytical method for nitroglycerin must be both sensitive (25-pg/mL detection limit) and specific (*i.e.*, must measure nitroglycerin in the presence of metabolites).

Several different analytical methods have been developed. HPLC methods have been developed to measure nitroglycerin amounts and concentrations in dosage forms (6), waste water (7), trace level explosives (8), and blood (9). Of these HPLC methods, the last (9) used a thermal energy analyzer–detector and was the most sensitive. This method was capable of detecting 500 pg (injected on column) and required 2 mL of plasma (CV = 12% at 13 ng/mL). None of these methods are capable of detecting nitroglycerin concentrations of 50 pg/mL.

Within the past few years, four GC–MS assays for nitroglycerin have been described (10–13). These assays possess some advantages over HPLC and conventional gas chromatographic (GC) assays. GC–MS assays are selective for the parent drug and quite sensitive (typically 50 pg/mL). Several disadvantages of GC–MS methods are readily apparent. The cost and time to set up a GC–MS instrument is often prohibitive for most clinical laboratories. Several of these assays (11–13) require the use of specially synthesized internal standards labeled with stable isotopes. These internal standards often are not commercially available, which may certainly limit the utility of these assays for routine clinical monitoring.

Several GC assays utilizing conventional packed columns have been developed (14–16). These were not fully satisfactory due to lack of sensitivity (14), complicated extraction procedure and questionable precision [*i.e.*, CV = 23% at 100 pg/mL (15)], and lack of specificity (16). Wu *et al.* (16) found interfering chromatographic peaks, which varied between individuals and even varied within individuals with time. Another major obstacle to the determination of picogram concentrations of nitroglycerin with conventional packed-column GC is adsorption of nitroglycerin to the column components. Several investigators have found that preloading columns, *i.e.*, injecting large amounts of nitroglycerin immediately before analyses of plasma samples, resulted in decreased column adsorption (17–19).

Drug adsorption to GC columns may be greatly reduced, if not eliminated, with the use of inert fused-silica capillary columns (20). Douse (20) reported a fused-silica capillary GC assay capable of detecting 5 pg and quantifying 100 pg of nitroglycerin (CV = 13%). Sved *et al.* (21) recently reported nitroglycerin plasma levels after a topical dose of nitroglycerin ointment. Although a detection limit of 50 pg/mL (using a fused-silica column) was reported, details of the assay validation have not been reported to date. In the present assay, the use of an on-column injector, a syringe with a fused-silica capillary needle, and fused-silica capillary columns allows the quantitation of picogram concentrations of nitroglycerin in plasma samples.

This report describes the capillary GC determination of nitroglycerin in plasma using on-column injection and electron-capture detection (EC). The assay described possesses the precision, sensitivity, and selectivity required to analyze picogram nitroglycerin concentrations in human plasma.

EXPERIMENTAL SECTION

Chemicals and Reagents—Nitroglycerin was obtained as a 10% triturate on lactose[1] and was extracted from the lactose with ether. The internal standard was 2,6-dinitrotoluene[2]. All solvents[3] (pesticide quality) were used as supplied, with the exception of heptane. Heptane was further purified with activated charcoal. Dimethyldichlorosilane[4] was commercially obtained.

Glassware Silanization Procedure—All glassware was silanized before use. A 5% (v/v) dimethyldichlorosilane solution in toluene was prepared fresh monthly and stored at 4°C. Glassware was soaked in this solution for 20 min, rinsed in toluene, and soaked in methanol for 20 min to neutralize excess reagent. The glassware was rinsed a final time with fresh methanol, dried at 100°C for 1 h, and baked at 260°C for 6 h.

[1] Key Pharmaceuticals, Inc., Miami, Fla.
[2] K & K Laboratories, Hollywood, Calif.
[3] Glass-distilled; Burdick and Jackson, Muskegon, Mich.
[4] Aldrich Chemical Co., Milwaukee, Wis.

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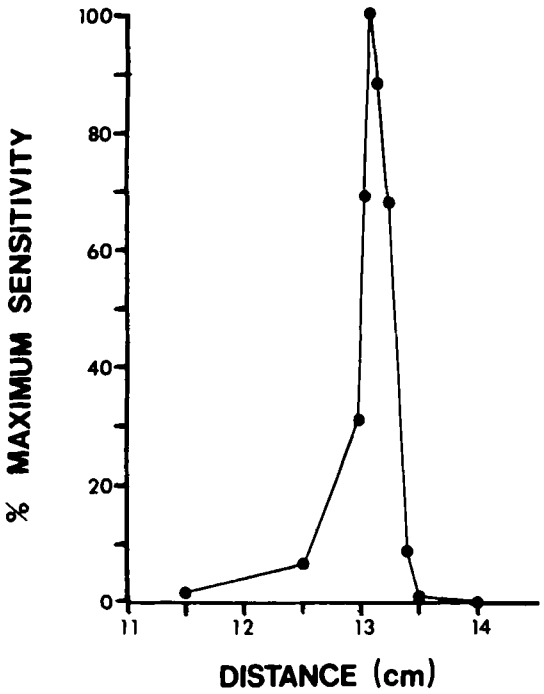

Figure 1—*Effect of capillary column alignment inside the GC detector* (i.e., *distance column is inserted through the detector insert) on nitroglycerin sensitivity.*

Instruments—The gas chromatographs[5] were equipped with ^{63}Ni electron-capture detectors (EC) and on-column injectors[6] (this assay has been validated on two separate instruments). The columns were 30 m × 0.25-mm fused silica capillary columns coated with a cross-linked methyl polysiloxane stationary phase[7]. The capillary column was wrapped in a single sheet of aluminum foil; this technique was found to eliminate peak splitting, which occurred due to uneven heat distribution within the GC oven. Helium was used as a carrier gas at a flow rate of 1.5 mL/min (inlet pressure = 40 psi). Nitrogen flow rates to the EC and column make-up were 5 and 25 mL/min, respectively. Column position inside the EC was optimized for maximum sensitivity. The detector temperature was maintained at 300°C. The oven temperature was programmed from 120°C to 180°C at 5°C/min. A second temperature ramp was used to "burn-off" the residual extract. This was accomplished by programming the oven at 50°C/min to 250°C and maintaining this final temperature for 10 min. Chromatograms were recorded and peak height ratios calculated using a recording integrator[8].

With routine use (every 100 injections) a 40-cm length of column (at the injector end) should be removed. This procedure, which results in more reproducible standard curves, removes nonvolatile extract components that accumulate and eventually cause chromatographic peaks to broaden and the sensitivity to decrease. An alternative to removing portions of the analytical column is the use of a precolumn. The precolumn is a 1-m section of column with the same liquid phase as the analytical column. The precolumn should also be wrapped in aluminum foil and connected directly to the injector. The opposite end of the precolumn is connected to the analytical column using a zero dead-volume union[9]. After ~100 injections, the precolumn may be changed and column efficiency is not lost.

Clinical Blood Sampling—Serial blood samples were drawn into chilled 7-mL evacuated tubes[10] through an indwelling venous catheter kept patent with heparin. The blood was immediately centrifuged at 12,800×*g* for 20 s. The plasma was then immediately frozen in a dry ice bath. The total amount of time required to withdraw the blood sample and centrifuge to obtain plasma prior to freezing was <2 min. This procedure minimizes the *in vitro* degradation of nitroglycerin by blood.

Sample Preparation—Nitroglycerin calibration standards were prepared as follows. Primary stock solutions (1 mg/mL each) of nitroglycerin and 2,6-dinitrotoluene were prepared in ethanol. Secondary stock solutions (10 μg/mL) were prepared fresh monthly by diluting the primary standards in ethanol (1:100, v/v). Fresh aqueous solutions of nitroglycerin (10 ng/mL) and 2,6-dinitrotoluene (20 ng/mL) in distilled water were prepared daily from the secondary stock solutions. Nitroglycerin calibration standards (0, 25, 50, 100, 200, 300, 400, 500, 750, 1000, and 2000 pg/mL) were prepared by adding the appropriate aliquot of the nitroglycerin standard and 50 μL (1 ng) of 2,6-dinitrotoluene to 1-mL samples of blank human plasma in 16 × 150-mm silanized test tubes with polytef-lined screw caps. Clinical plasma samples (1 mL) were transferred to silanized test tubes and mixed with 50 μL of the 2,6-dinitrotoluene.

Each calibration standard or clinical sample was extracted with 10 mL of pentane by vortexing for 2 min, followed by centrifugation for 10 min at 2000×*g*. The pentane layers were transferred to clean test tubes. The residual plasma layers were extracted and centrifuged a second time, as described above. The combined pentane extracts were evaporated under a stream of nitrogen (at room temperature) to a final volume of ~1 mL. The extracts were then transferred to 1-mL vials[11] and evaporated to dryness under a stream of nitrogen. Immediately after the solvent had been completely removed, 25 μL of *n*-heptane was added to each vial, and the vials were vortexed (~5 s). A 1.0-μL aliquot of each extract was injected into the gas chromatograph.

Sample application to the column was performed *via* the injector stopcock using a 10-μL gas-tight syringe which was modified to hold a 0.21 × 190-mm fused-silica capillary "needle." All extracts were stored at −20°C until chromatographed and on dry ice between GC injections.

Assay Specificity—Assay specificity was determined by injecting solutions of several drugs and some of their known metabolites on column. The oven temperature was programmed from 120°C to 200°C at a rate of 5°C/min, then ballistically to 250°C. This temperature program is only slightly different than that described earlier and will not affect the retention time of nitroglycerin, nitroglycerin metabolites, or the internal standard. This temperature program was used to show the relative retention times of a related drug, isosorbide dinitrate, and it metabolites. The analytical method described may be modified in this manner to determine plasma isosorbide dinitrate and metabolite concentrations. All of the compounds tested for possible interferences were tested using these same conditions.

Nitroglycerin Stability—Nitroglycerin stability in frozen human plasma was evaluated at −20°C. Drug-free plasma was obtained from four volunteers

Table I—Retention Times of Nitroglycerin, Nitroglycerin Metabolites, and Several Other Commonly Prescribed Drugs

Compound	GC Retention Time, min
Nitroglycerin	8.6
1,3-Dinitroglycerin	7.5
1,2-Dinitroglycerin	7.6
1-Glycerylmononitrate	4.5
2-Glycerylmononitrate	4.7
2,6-Dinitrotoluene	11.4
Isosorbide dinitrate	14.5
Isosorbide-2-mononitrate	9.8
Isosorbide-5-mononitrate	12.2
Acetaminophen	ND[a]
Acetylsalicylate	11.9
Caffeine	ND
Chloral Hydrate	1.0
Furosemide	ND
Hydrochlorothiazide	ND
Metolazone	ND
Procainamide	ND
N-Acetylprocainamide	ND
Spironolactone	ND
Triamterene	ND

[a] ND = not detected.

Table II—Within-Day Nitroglycerin Assay Precision and Accuracy

Nitroglycerin Concentrations, pg/mL			
	Calculated		
Actual	Mean ± *SD*[a]	*CV*, %	Error, %
25	21.3 ± 1.6	7.6	−16
50	49 ± 3	6.1	−2
100	104 ± 2	2.3	+4
300	288 ± 6	2.1	−4
1,000	1015 ± 39	3.8	+2
10,000[b]	9890 ± 670	6.8	−1

[a] $n = 6$. [b] Diluted 1:10 (v/v) before extraction.

[5] Varian 3700 and 6000 gas chromatographs; Varian Associates, Los Altos, Calif.
[6] On-column injector; J & W Scientific, Inc., Rancho Cordova, Calif.
[7] DB-1, 1.0-μm film thickness; J & W Scientific, Inc., Rancho Cordova, Calif.
[8] HP3390A; Hewlett Packard, Palo Alto, Calif.
[9] Zero dead volume union; J & W Scientific, Inc., Rancho Cordova, Calif.
[10] Vacutainers; Becton, Dickenson and Co., Rutherford, N.J.
[11] Reacti-vials; Pierce Chemical Co., Rockford, Ill.

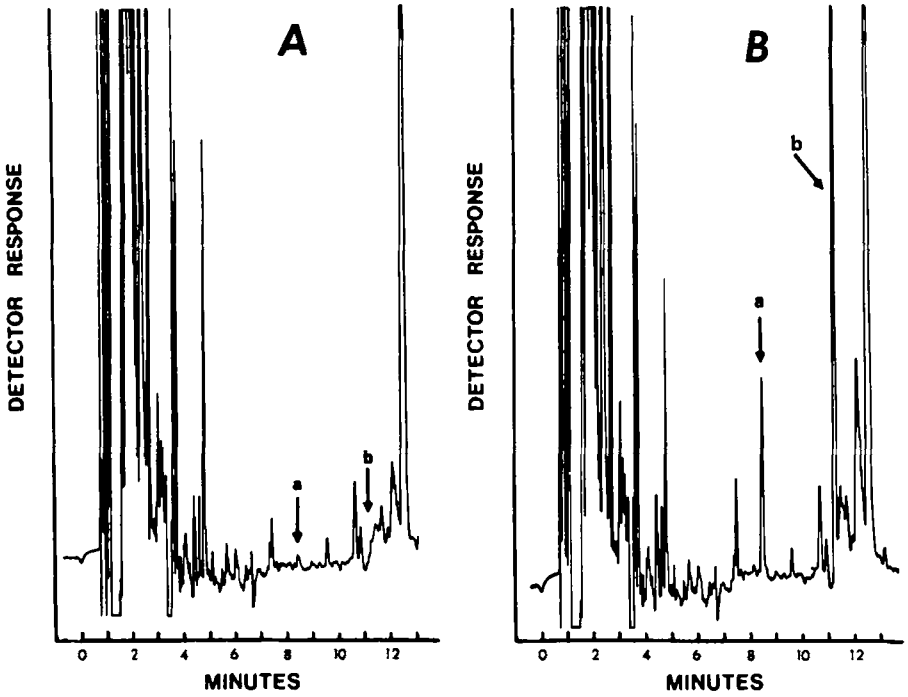

Figure 2—*Chromatograms of blank and nitroglycerin-fortified plasma extracts. Key: (A) blank plasma extract; (B) plasma extract fortified with 100 pg/mL of nitroglycerin and 1 ng/mL of 2,6-dinitrotoluene. Retention times for nitroglycerin and 2,6-dinitrotoluene are indicated in each chromatogram by* a *and* b, *respectively.*

(three male, one female) and pooled. Nitroglycerin was added to two separate 100-mL aliquots of plasma (at 0°C) to obtain final concentrations of 0.1 and 1.0 ng/mL. Each plasma solution was subdivided into multiple 1-mL aliquots, quick-frozen in dry ice, and stored at −20°C. Six aliquots of each concentration were assayed (as described above) after 0, 7, 14, 24, 40, and 60 d of storage.

Column/Electron-Capture Detector Optimization—The position of the capillary column exit inside the detector was optimized for maximum sensitivity. Sensitivity was evaluated from multiple injections of a 1.0-μL aliquot of a nitroglycerin standard solution (1.0 pg of nitroglycerin/μL and 40 pg of 2,6-dinitrotoluene/μL, in heptane). The position of the column in the detector was adjusted to increase sensitivity (maximum nitroglycerin/2,6-dinitrotoluene peak height ratio).

RESULTS AND DISCUSSION

Injector and Detector Optimization—Initial attempts to use a capillary GC system to quantitate picogram amounts of nitroglycerin were unsuccessful. Nitroglycerin may adsorb and degrade on active surfaces of capillary injectors, glass columns, and detectors. Several injection systems were evaluated. Significant nitroglycerin adsorption occurred with splitless and direct injectors[12]. Use of the split injection technique (90:1 split ratio) decreased the apparent adsorption of nitroglycerin to the injector. Lower split ratios were necessary to increase assay sensitivity, but the coefficient of variation was >20%. Finally, injector precision and assay sensitivity were evaluated using the on-column injection technique. Use of this technique allowed nitroglycerin to be applied directly onto the capillary column, thus avoiding glass and metal surfaces which may adsorb and/or degrade nitroglycerin.

Adsorption and degradation of nitroglycerin may occur not only on surfaces of conventional injectors, but also on active surfaces present in the EC. An advantage of using narrow-bore fused-silica capillary columns is that the column may be inserted entirely into the EC. Optimization of the position of the column exit inside the EC is an important determinant of assay sensitivity. The EC contains highly active surfaces which may bind or degrade labile drugs such as nitroglycerin. Therefore, the column must be positioned to both minimize nitroglycerin loss and maximize detector sensitivity.

Figure 1 shows that detector sensitivity sharply increases as the column is inserted further into the EC and peaks at 13.1 cm, *i.e.*, 13.1 cm as measured from the EC detector insert to the end of the column. Further insertion of the column into the detector results in a sharp decrease in sensitivity. Note that if the column alignment varies by as little as 1–2 mm, 50% of the maximum sensitivity is lost.

The position of the column within the EC may not be as critical in the chromatography of other compounds. For example, lindane and aldrin are easily detected even when the column outlet is placed only 0.5 cm inside the detector insert.

Extraction Precision and Recovery—Initial assay development utilized an extraction procedure in which 1.0 mL of plasma was extracted once with 10 mL of pentane. Extraction efficiencies for nitroglycerin and 2,6-dinitrotoluene (1.0 ng/mL each) were 75 ± 7% and 93 ± 2%, respectively. When biological samples (plasma) were each extracted twice, assay precision, as measured by the decrease in coefficient of variation, was increased by two- to threefold. Coefficients of variation decreased from 13.7 to 6.1%, 15.9 to 2.3%, and 9.5 to 2.9% at plasma nitroglycerin concentrations of 50, 100, and 1000 pg/mL, respectively, when single and double extractions are compared.

Nitroglycerin Chromatography and Assay Specificity—Chromatograms of blank and nitroglycerin-fortified plasma are shown in Fig. 2. Figure 2A depicts a chromatogram for a blank plasma extract. The nitroglycerin and 2,6-dinitrotoluene retention times are indicated by arrows. The small peak, indicated by arrow a, has the same retention time as nitroglycerin. This peak is due to a contaminant present in the heptane and was eliminated by purifying the heptane with activated charcoal. Figure 2B shows a representative chromatogram of plasma fortified with 100 pg/mL of nitroglycerin and 1 ng/mL of 2,6-dinitrotoluene. The retention times for nitroglycerin and 2,6-dinitrotoluene were 8.6 and 11.4 min, respectively.

Both nitroglycerin and dinitrotoluene were resolved from nitroglycerin metabolites. The retention times for the metabolites are shown in Table I. Since the capillary GC assay will be used to measure nitroglycerin plasma concentrations in patient populations, several other drugs were tested for assay interference. Table I lists the retention times, when detected, of several drugs (and metabolites) which may be prescribed to patients in a hospital cardiac care unit. None of the drugs, nor the metabolites tested, interfered with the nitroglycerin capillary GC assay. Also note that nitroglycerin may be assayed in the presence of isosorbide dinitrate, which is often concurrently administered to patients. No interference was noted for this compound and its two major metabolites, isosorbide-5-mononitrate and isosorbide-2-mononitrate.

Assay Precision, Accuracy, and Linearity—The precision and accuracy of the nitroglycerin assay procedure was assessed by analysis of six replicate

Table III—Stability of Nitroglycerin in Pooled Human Plasma at 100- and 1000-pg/mL Concentrations When Stored at −20°C

	Calculated Concentration, pg/mL			
	100 pg/mL		1000 pg/mL	
Time, d	Mean ± *SD*	*CV*, %	Mean ± *SD*	*CV*, %
0	112 ± 7	6.2	1015 ± 39	3.8
7	102 ± 5	4.9	1034 ± 31	3.0
14	109 ± 7	6.4	987 ± 31	3.1
24	102 ± 4	3.9	933 ± 33	3.5
40	113 ± 6	5.3	952 ± 31	3.4
60	106 ± 6	5.7	955 ± 41	4.2

[12] Varian Associates, Los Altos, Calif.

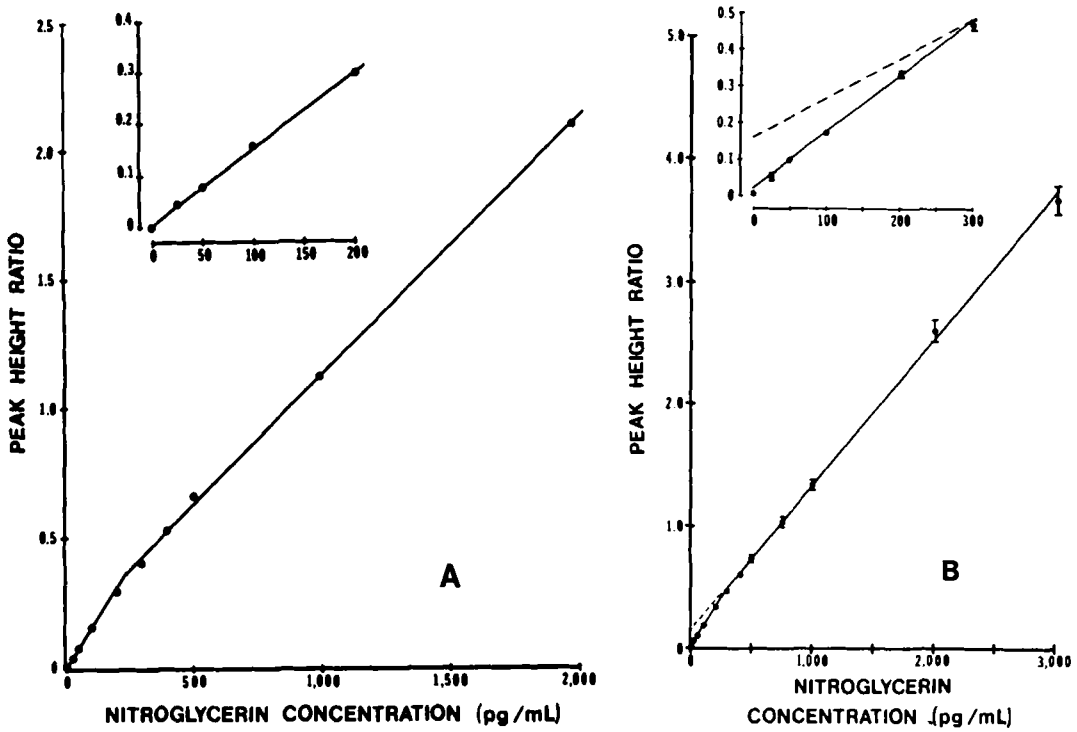

Figure 3—*Nitroglycerin standard curves. Key: (A) Representative nitroglycerin standard curve; curve is linear between 0–200 pg/mL and 200–2000 pg/mL. Inset shows the low calibration standard range. (B) Mean of six individual nitroglycerin standard curves. Standard error bars are shown except when smaller than the data symbol. Inset shows the low calibration standard range and the extrapolated line (- - -) from the high concentration range.*

plasma samples to which known amounts of nitroglycerin were added. Within-day assay precision and accuracy are summarized in Table II. Coefficients of variation over the concentration range, 25–10,000 pg/mL, were <10%. The relatively high coefficient of variation of the 10-ng/mL concentration may be due, in part, to the additional steps involved in sample analysis at higher concentrations, *i.e.*, extracting 100 μL of plasma diluted with 900 μL of blank plasma. Accuracy for all concentrations, except 25 pg/mL, was within ±5% of the actual fortified nitroglycerin concentrations. Between-day assay precision was evaluated over 2 months (40 standard curves), *i.e.*, each day for 40 d. Blank plasma samples were fortified with 0.1 and 1.0 ng/mL of nitroglycerin. These samples served as calibration controls until drug stability could be established. Coefficients of variation were 7 and 12% at 1.0 and

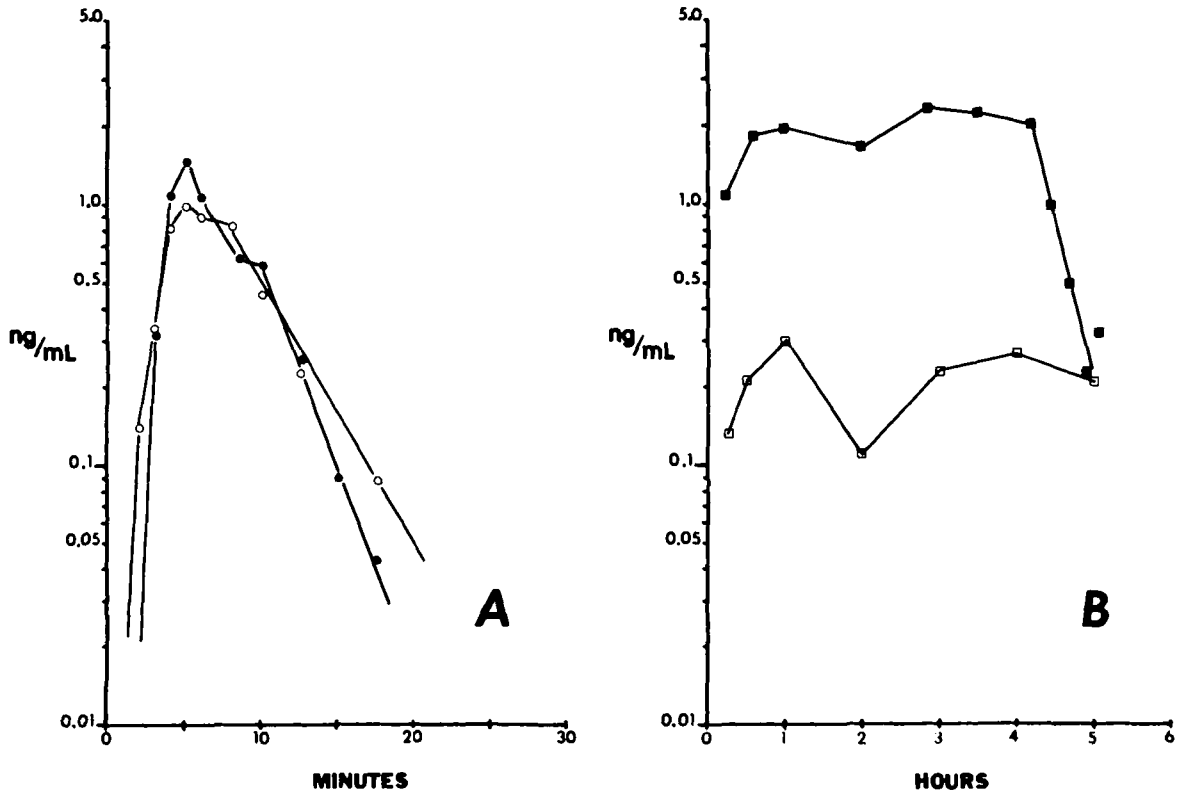

Figure 4—*Plasma nitroglycerin concentration* versus *time profiles for two subjects. Key: (A) after 0.4-mg sublingual doses to subject 1; (B) after topical doses to subject 2* via *ointment (■) and controlled-release patch (□).*

0.1 ng/mL, respectively. Accuracy (mean concentrations) were within the percent error noted for these two concentrations in Table II.

Nitroglycerin standard curves were not linear over the entire concentration range of 0–3000 pg/mL. At very low concentrations (<200–300 pg/mL) the standard curve slope was generally 50% greater than that of the high concentration range. Figure 3A shows a representative standard curve. The change in slope is not due to extraction efficiencies, since the slope change was also present when known concentrations of drug in heptane were injected. The "break-point" of the standard curve was consistent and did not change during any given day. Even when six different standard curves were averaged together (Fig. 3B), the break-point was still present. Therefore, this break-point is not due to assay variability.

The break-point may vary between different columns, instruments, or make-up gas flow rates. The standard curve in Fig. 3A was analyzed on a different instrument than the curves averaged in Fig. 3B. The break-point in Fig. 3A occurred at 200 pg/mL. In each of the six individual curves and the mean curve in Fig. 3B, the break-point occurred at 300 pg/mL. The position of the break-point may vary between instruments, but is constant within any one instrument. Although the occurrence of the break-point is quite consistent, the cause has not yet been identified.

Nitroglycerin Stability in Frozen Plasma—Nitroglycerin stability in frozen plasma has been determined in some detail by Maier *et al.* (22). Because these investigators determined nitroglycerin concentrations using a GC assay which utilized packed columns, it was necessary to validate the plasma storage conditions using the capillary GC assay reported here. The nitroglycerin stability data are shown in Table III. Nitroglycerin was stable in plasma for a minimum of 60 d when stored at −20°C.

Clinical Studies—As an illustration of the applicability of this assay procedure, nitroglycerin was administered to two healthy volunteers. Subject 1 received a 0.4-mg sublingual dose on two separate occasions (1 week apart); the plasma concentrations are shown in Fig. 4A. Nitroglycerin peak times were 5 min, and terminal half-lives were 2.0 and 3.0 min. Subject 2 received two topical doses of nitroglycerin on separate occasions. The subject received a 1-g dose of 2% nitroglycerin ointment (equivalent to 20 mg of nitroglycerin) which was spread evenly over a 200-cm^2 chest area and occluded. The second dose was a sustained-release nitroglycerin patch. Plasma concentrations are shown in Fig. 4B. The half-life (15 min) after removing the ointment was longer than that after sublingual dosing. The longer half-life is most likely due to continued absorption of nitroglycerin.

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