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[x] To whom inquiries should be directed.

Electron-Capture GLC Determination of Nanogram to Picogram Amounts of Isosorbide Dinitrate

J. O. MALBICA [x], K. MONSON, K. NEILSON, and R. SPRISSLER

Abstract □ A GLC method for the determination of plasma isosorbide dinitrate using electron-capture detection is described. The organic nitrates are especially suited for electron-capture detection if the detector temperature is optimized for maximum sensitivity, *e.g.*, 175°. Proper maintenance of the detector and column assures reproducible data in the low nanogram range. The extraction procedure described is simple, efficient, and expedient for processing large numbers of samples. The method was used to study plasma levels in four human volunteers after a single dose of a 5-mg chewable isosorbide dinitrate tablet. Concentration levels of isosorbide dinitrate as low as 0.5 ng/ml of plasma can be measured by this procedure.

Keyphrases □ Isosorbide dinitrate—electron-capture GLC analysis, human plasma □ GLC, electron capture—analysis, isosorbide dinitrate, human plasma □ Vasodilators, coronary—isosorbide dinitrate, electron-capture GLC analysis, human plasma

The low dosage of isosorbide dinitrate sufficient for pharmacological effectiveness results in low circulating blood levels of the drug, and difficulties exist in assaying for low nanogram to picogram amounts. Previously described GLC methods (1, 2) were either laborious or not sensitive enough to follow rapidly declining blood levels of isosorbide dinitrate.

Since only limited data are available on the blood levels of isosorbide dinitrate following administration of therapeutic doses, a method was needed that would allow reliable estimations of blood isosorbide dinitrate levels in the low nanogram to picogram range using conventional electron-capture GLC instrumentation. This report describes an assay method for isosorbide dinitrate that is expedient, sensitive, and reproducible for routinely handling large numbers of samples.

EXPERIMENTAL

Reagents and Materials—Benzene[1] and ethyl acetate[1] were glass-distilled reagent grade solvents. Anhydrous sodium sulfate[2] was reagent grade, ACS certified. Charcoal-treated paper disks[3], 6.5-mm diameter, were made with a paper punch-out.

One hundred grams of anhydrous sodium sulfate powder was washed with benzene for 20 min, allowed to air dry, and then placed in an oven at 100° until needed.

The charcoal-treated paper disks were similarly washed in benzene. Excess benzene was removed, and the paper disks were allowed to air dry. The disks were stored in an air-tight container until required.

Isosorbide dinitrate[4] and isoidide dinitrate[4] were purified by recrystallization from ethanol.

Plasma Level Study—Isosorbide dinitrate was administered to four healthy volunteers (one male, age 34; three females, ages 23, 25, and 27); each received a 5-mg chewable isosorbide dinitrate tablet[5]. Blood, 10 ml, was drawn from the cubital vein into a heparinized tube from each subject 0, 15, 30, 60, 120, and 180 min following isosorbide dinitrate administration. The blood was immediately centrifuged at 3000 rpm in an angular head clinical centrifuge[6] for 15 min. The plasma was collected and stored at −10° prior to analysis.

Extraction of Isosorbide Dinitrate—Five-milliliter aliquots of benzene were added to a 15.0-ml graduated conical centrifuge tube containing 1.0 ml of plasma spiked with 10 ng of isoidide dinitrate as an internal standard. The tube was mixed on a vortex[7] for 1 min and centrifuged at 2000 rpm to separate the phases. Then the benzene phase was carefully removed to another 15-ml centrifuge tube. A 0.5-g portion of benzene-washed sodium sulfate and three benzene-washed charcoal-treated paper disks were added to the benzene extract.

The tube and its contents were again vortexed for 15 sec and centrifuged as previously described. As much of the benzene layer as possible was carefully removed to another centrifuge tube and dried completely under a nitrogen stream (using an in-line activated silica gel desiccant filter). To each residue was added 100 μl of ethyl acetate just prior to injection. Samples to be analyzed at a later time, *i.e.*, up to 24 hr, were stored, covered tightly, and protected from light.

Electron-Capture GLC Detection—The prepared GLC samples were analyzed using a gas chromatograph[8] equipped with a ^{63}Ni-electron-capture detector. A glass column (120 cm × 4 mm i.d.) was packed with 3% QF-1 on Gas Chrom Q, 100–200 mesh. The injection port and oven temperatures were maintained at 210 and 150°, respectively. The detector was set at 175° and operated with a pulse interval of 150 μsec. The flow rate of the carrier gas, argon with 5% methane, was 95 ml/min. Under these conditions, the retention times of isoidide dinitrate and isosorbide dinitrate were 2.0 and 3.0 min, respectively. The injection volume was 5 μl, and the injection was performed in duplicate.

Calculations—A stock solution of isosorbide dinitrate in absolute ethyl alcohol (1 μg/ml) was used to spike plasma in the 5–75-ng/ml range. A stock solution of isosorbide dinitrate (0.5 μg/ml) was used to spike plasma in the 0–5-ng/ml range. The samples were likewise spiked with 10 ng of isoidide dinitrate as an internal standard. A response factor (peak area ratio) was calculated for the standard. The concentration of isosorbide dinitrate in the samples was determined from the standard's response factor.

Extraction Efficiency—The overall recovery of isosorbide dinitrate by the described extraction procedure was determined by spiking plasma

[1] Burdick-Jackson Labs., Bodman Chemicals, Narberth, Pa.
[2] J. T. Baker Chemical Co., Phillipsburg, N.J.
[3] S & S charcoal-treated filter paper No. 505.
[4] Stuart Pharmaceutical Division, ICI United States Inc.
[5] Sorbitrate, Stuart Pharmaceutical Division, ICI United States Inc.
[6] International Equipment Co., Needham, Pa.
[7] Vortex Genie, Fisher Scientific.
[8] Hewlett-Packard HP 7620A.

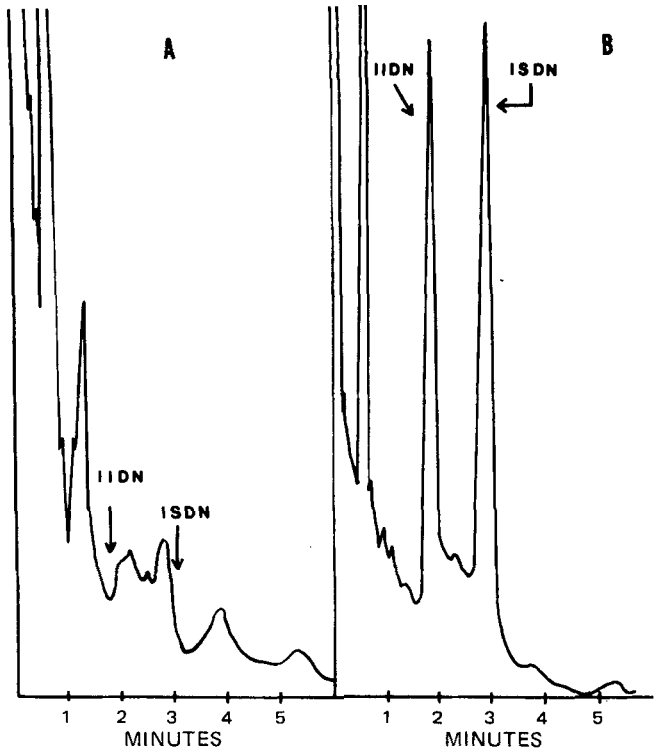

Figure 1—*Typical gas chromatograms obtained from blank plasma (A) and plasma containing 15 ng of isosorbide dinitrate/ml (B). Key: ISDN, isosorbide dinitrate; and IIDN, isoidide dinitrate.*

samples in triplicate with 20 μl of ^{14}C-isosorbide dinitrate (18 μCi/mg, 4.5 μg/ml). Likewise, a triplicate set of plasma samples was spiked with 5 and 10 μl of ^{14}C-isosorbide dinitrate solution (18 μCi/mg, 0.45 μg/ml). The samples were extracted as previously described and dried under nitrogen. The residue was dissolved in 1.5 ml of ethyl acetate, and 1.0 ml was counted to determine the ^{14}C-isosorbide dinitrate content. The recovered ^{14}C-isosorbide dinitrate was compared with the actual amount of ^{14}C-isosorbide dinitrate added.

RESULTS AND DISCUSSION

A typical gas chromatogram of isosorbide dinitrate extracted from plasma is shown in Fig. 1B; the chromatogram obtained from blank plasma is shown in Fig. 1A. The lower limit of detection was 0.5 ng/ml and was reproducible under the experimental conditions.

Table I—Response Ratios[a] (Day-to-Day Variations in the Mean)

Isosorbide Dinitrate Concentration, ng/ml of Plasma	Day 1		Day 2		Day 3	
	Mean[b]	CV[c]	Mean	CV	Mean	CV
1	0.10 ± 0.02	17	0.11 ± 0.05	49	0.08 ± 0.01	11
3	0.19 ± 0.03	15	0.14 ± 0.07	49	0.15 ± 0.01	6
5	0.26 ± 0.03	14	0.25 ± 0.05	19	0.21 ± 0.08	38
10	0.45 ± 0.07	16	0.39 ± 0.13	33	0.50 ± 0.18	40
25	1.05 ± 0.17	16	1.02 ± 0.17	17	0.96 ± 0.10	10
50	2.01 ± 0.21	10	1.94 ± 0.09	5	2.12 ± 0.14	7
75	3.35 ± 0.19	6	2.64 ± 0.27	10	3.14 ± 0.15	5

[a] The area of isosorbide dinitrate was divided by the area of isoidide dinitrate. [b] Mean ± *SD* based on a quadruplicate plasma run for each concentration value. [c] *CV* = coefficient of variation = (σ/mean) × 100.

Table II—Effect of Electron-Capture Detector Temperature on Peak Height for a Total of 4 ng of Isosorbide Dinitrate Injected onto the Column

Detector Temperature	Peak Height, cm
170°	4.178
175°	4.882
180°	4.545
185°	4.415
197°	4.512
210°	3.388
227°	3.041

Table III—Human Plasma Levels of Isosorbide Dinitrate after Administration of a 5-mg Chewable Isosorbide Dinitrate Tablet

Subject (Sex, Age)	Plasma Isosorbide Dinitrate Level, ng/ml					
	0	15 min	30 min	60 min	120 min	180 min
F.B. (M, 34)	N.D.[a]	2.4	1.9	0.9	N.D.	N.D.
J.F. (F, 23)	N.D.	5.6	2.8	1.4	N.D.	N.D.
K.M. (F, 27)	N.D.	5.3	1.2	0.6	N.D.	N.D.
K.N. (F, 25)	N.D.	3.7	2.6	N.D.	N.D.	N.D.
Total	N.D.	4.3 ± 1.3	2.1 ± 0.6	0.7 ± 0.4	N.D.	N.D.

[a] N.D. = not detected.

The results from quadruplicate runs on plasma spiked with varying amounts of isosorbide dinitrate are summarized in Table I where the day-to-day mean, standard deviation, and coefficient of variation are given. The overall coefficient of variation of the procedure in the 1–75-ng/ml range was less than 20% except in the 1–10-ng/ml range where, as expected, it sometimes increased with decreasing concentration.

A plot of the response ratio *versus* concentration showed that the described procedure was reproducible from day to day and that linearity was established throughout the 0–75-ng/ml range.

Isosorbide dinitrate was stable when stored frozen in plasma up to 1 week. Isosorbide dinitrate can be extracted from plasma and stored as the dry residue for at least 24 hr.

To obtain maximum sensitivity for the GLC measurement of isosorbide dinitrate, the optimal detector response with temperature was determined (Table II). The detector produced the maximum response for a given quantity of isosorbide dinitrate (4 ng) in the 175–200° temperature range. It is suggested that the optimal electron-capture detector temperature be determined for each GLC unit. The detector temperature was raised to 350° overnight to eliminate the accumulation of any plasma-derived components as a result of operating at a lower temperature during the assay.

Priming the column was essential to achieve high sensitivity. Several 1-μl injections of an isosorbide dinitrate solution (10 ng/ml) were made until a constant peak height for isosorbide dinitrate in the chromatogram was obtained.

After a period of weeks, as a result of numerous injections, a gradual

Table IV—Overall Recovery of ^{14}C-Isosorbide Dinitrate Added to Human Plasma

Isosorbide Dinitrate, ng/ml	Disintegrations per Minute Added	Disintegrations per Minute Recovered	Recovery, % ± *SD*
2.2	99	81	81.8
2.2	99	79	79.8
2.2	99	78	78.8
		Average	80.1 ± 1.2
4.5	192	147	76.6
4.5	192	141	73.4
4.5	192	144	75.0
		Average	75.0 ± 1.3
90.0	3621	2752	76.0
90.0	3621	2754	76.1
90.0	3621	2732	75.4
		Average	75.8 ± 0.3

loss in sensitivity was observed as well as the appearance of interfering peaks in the chromatogram. At this time, a new column was installed and primed.

The isosorbide dinitrate human plasma levels following a 5-mg dose of chewable isosorbide dinitrate were highest at 15 min after administration in all four subjects (Table III). No isosorbide dinitrate levels were detectable at 120 min or more after administration.

The overall recovery of ^{14}C-isosorbide dinitrate in the 2.2–90-ng/ml concentration range is shown in Table IV. The recovery of 75–80% was independent of concentration.

Previous attempts to quantitate isosorbide 2-mononitrate and isosorbide 5-mononitrate in the same concentration range as isosorbide dinitrate were unsuccessful. Certain plasma components that appeared in the benzene extract from time to time interfered with the determination of the mononitrates of isosorbide dinitrate.

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[x] To whom inquiries should be directed.

Potency of Synthetic Luteinizing Hormone Releasing Hormone Preparations in Rat Anterior Pituitary Cell Cultures

W. C. DERMODY [x], C. A. PASTUSHOK, R. SAKOWSKI, J. W. VAITKUS, and J. R. REEL

Abstract □ Selected synthetic luteinizing hormone releasing hormone preparations were assayed, and their potencies were determined relative to one sample utilizing primary cultures of enzymatically dispersed rat anterior pituitary cells. Preliminary cell culture experiments indicated that luteinizing hormone releasing hormone had to be in constant contact with cells for continued luteinizing hormone secretion. Luteinizing hormone levels in media reached a maximum concentration after 4 hr of continuous luteinizing hormone releasing hormone exposure. Cell culture bioassay was selected over the bioassay employing chronically ovariectomized steroid-blocked rats due to greater sensitivity and economy. The assay of each luteinizing hormone releasing hormone preparation was replicated four to seven times. Preparations from several companies were less potent ($p < 0.05$) than the reference product. Contaminants were disclosed by TLC in preparations with potencies lower than the reference product.

Keyphrases □ Luteinizing hormone releasing hormone—various synthetic preparations analyzed by TLC and radioimmunoassay, potencies determined, rat anterior pituitary cells □ TLC—analysis, luteinizing hormone releasing hormone, various synthetic preparations □ Radioimmunoassay—analysis, luteinizing hormone releasing hormone, various synthetic preparations □ Hormones—luteinizing hormone releasing hormone, various synthetic preparations analyzed by TLC and radioimmunoassay, potencies determined, rat anterior pituitary cells

Although luteinizing hormone releasing hormone has been synthesized in many laboratories, no potency comparisons of various synthetic preparations have been published. However, it was reported that luteinizing hormone releasing hormone, synthesized by several laboratories (1–3), exhibited biological activity equivalent to an isolated natural porcine luteinizing hormone releasing hormone preparation[1]. These results were obtained utilizing either short-term incubations of minced pituitary tissue or ovariectomized estrogen–progesterone-blocked rats. This report presents potency comparisons of various synthetic luteinizing hormone releasing hormone preparations assayed with cultures of rat anterior pituitary cells.

EXPERIMENTAL

Various synthetic luteinizing hormone releasing hormone preparations (Samples 1–6[2–6]) were obtained commercially. These preparations were compared to Sample 7[7] luteinizing hormone releasing hormone.

The reference product (Sample 7) was prepared by fragment condensation and purified by partition chromatography[8] with 1-butanol[9]–acetic acid–water (4:1:5, upper phase). The major fraction from the partition column was homogeneous in the following TLC systems on silica gel 60 F-254 glass plates[10]: System 1, chloroform–methanol–water (45:45:10); System 2, chloroform–methanol–water–acetic acid (60:45:10:1); System 3, chloroform–methanol–32% acetic acid (60:45:20); System 4, 1-butanol–acetone–water–acetic acid–5% ammonium hydroxide (45:15:20:10:10); System 5, 2-propanol–1 *M* acetic acid (2:1); System 6, 1-butanol–acetic acid–water (upper phase) (4:1:5); System 7, pyridine–ethyl acetate–acetic acid–water (5:5:1:3); and System 8, 1-butanol–pyridine–acetic acid–water (30:20:6:24).

The reference product corresponded to 89% peptide content (1.4 acetate salt, 3.5 hydrates) with a molecular weight of 1329.6 and $[\alpha]_D^{25} - 55.8°$ (c, 1 in 1% CH_3CO_2H)[11].

Molecular weights and peptide contents (Table I) of comparative luteinizing hormone releasing hormone preparations were obtained from package inserts or the company of origin. Peptide content varied from 80 to 88%. The purity of these preparations was examined using TLC System 2. Preliminary experiments indicated that System 2 was superior

[1] AVS 77-35, 215-269.

[2] Sample 1, lot 208052, and Sample 2, lot 221040, Spectrum Medical Industries, Torrance, Calif.

[3] Sample 3, lot A0402, Beckman Instruments, Palo Alto, Calif.

[4] Sample 4, lot 4562, Bachem, Marina Del Rey, Calif.

[5] Sample 5, lot 19-192AL, Abbott Laboratories, North Chicago, Ill.

[6] Sample 6, lot SY-1, Sankyo Co., Tokyo, 140 Japan.

[7] Sample 7, lot 10746 X71A, Parke, Davis & Co., Ann Arbor, Mich.

[8] Sephadex G-25 coarse, Pharmacia Fine Chemicals, Piscataway, N.J.

[9] All analytical reagents, Mallinckrodt, St. Louis, Mo.

[10] EM Laboratories, Elmsford, N.Y.

[11] Model 141, Perkin-Elmer Corp., Norwalk, Conn.