Journal of Chromatography, 413 (1987) 91–99
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3338

GAS CHROMATOGRAPHIC ASSAY OF GLYCEROL MONONITRATES IN BIOLOGICAL SAMPLES

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(First received April 16th, 1986; revised manuscript received July 17th, 1986)

SUMMARY

A new gas chromatographic analysis of glycerol 1-nitrate and glycerol 2-nitrate is described. The method is suitable for a variety of biological samples and can detect down to the low nanogram range. An extract of the sample to be analysed is treated with phenylboronic acid. The glycerol mononitrates rapidly form cyclic boronates, with five- and six-membered rings, respectively, which can then be separated by gas chromatography and detected by an electron-capture detector.

INTRODUCTION

Glycerol mononitrates are formed by denitration of glycerol trinitrate in the organism [1]. Recently it has been shown in animals that both isomers, glycerol 1-nitrate (G-1-N) and glycerol 2-nitrate (G-2-N), exert pharmacological effects that are qualitatively the same as those of the parent substance [2]. These mononitrates are therefore interesting for possible therapeutic use as vasodilators. More detailed studies are now required, in which the mononitrates either occur as breakdown products of nitroglycerin or are directly administered, and for these a convenient assay in biological samples had to be developed.

Previously published procedures for the analysis of glycerol mononitrates have used either thin-layer chromatography [3–5] or high-performance liquid chromatography (HPLC) [6–10]. Detection was by spectrophotometry [3], which suffers from lack of sensitivity, or by radiodetection of the ^{14}C-labelled compounds, which is expensive and time-consuming, or by thermal energy analysis [9,10], which is both extremely expensive and of very restricted application.

There are only a few reports on the use of gas chromatography (GC) for the analysis of glycerol mononitrates [11,12]. To our knowledge, in only one study, in which capillary GC was used, was resolution of the two mononitrates demonstrated [13]. However, the investigators were primarily interested in the assay

0378-4347/87/$03.50

of glycerol trinitrate and only wanted to show that there was no interference from the di- and mononitrates of glycerol.

We found that the GC of glycerol mononitrates is complicated by the poor thermal stability and volatility of these compounds. Even though we could achieve sufficient separation of the isomers on capillary as well as on packed columns, the reproducibility of the GC analyses remained unsatisfactory.

Alkyl and arylboronic acids and boronic anhydride react rapidly with diols and polyols, forming cyclic boronates [14]. For example, with carbohydrates this reaction is much more rapid than acetylation with acetic anhydride [15]. Five- and six-membered rings are preferably formed, as has been demonstrated by mass spectrometry [16]. These ring products are thermally stable and volatile, and hence are widely used as derivatives for the GC identification and assay of diols and polyols.

This paper describes an assay for the isomers of glycerol mononitrates in biological samples that employs phenylboronic acid as derivatizing reagent. Although in this report we emphasize the analysis of G-1-N, with G-2-N as internal standard, the method is also suitable for the analysis of G-2-N or of both isomers simultaneously.

EXPERIMENTAL

Instruments

A Packard Model 430 gas chromatograph equipped with a 10-mCi ^{63}Ni electron-capture detector was used throughout. The gas was purified over a filter (Chrompack) to remove oxygen. The glass columns (1.2 m$\times$2 mm I.D.) were silanized by treatment with a solution of 5% (v/v) dichlorodimethylsilane in toluene. The columns were rinsed with methanol, dried at 60°C for 2 h, then loaded with 3% SP 2401 on Supelcoport 100–200 mesh (Supelco, Sulzbach, F.R.G.).

IR spectra were obtained on a Bruker IFS 85 Fourier Transform IR spectrometer, with the samples incorporated in potassium bromide discs. ^{1}H NMR spectra were recorded on a Varian EM 360 spectrometer with tetramethylsilane as internal standard. Mass spectra were recorded on a Finnigan 4510 gas chromatograph–mass spectrometer, equipped with an Incos 2000 data system. Electron-impact spectra were obtained with an emission current of 210 μA, an ionization energy of 70 eV, an electron multiplier voltage of 1250 V and an ionizer temperature of 130°C.

Materials

G-1-N and G-2-N were synthesized in our laboratories. Phenylboronic acid was obtained from Fluka (Neu Ulm, F.R.G.) and activated charcoal from Chrompack (Mühlheim, F.R.G.). The solvents diethyl ether (Merck, Darmstadt, F.R.G.) and tetrahydrofuran (THF) (Fluka) were glass-distilled before use.

Synthesis and structural analysis of the derivatives

2-Phenyl-1,3,2-dioxaborinan-5-ol nitrate. Phenylboronic acid (0.61 g, 50 mmol) was dissolved in 20 ml of dry THF in a 50-ml round-bottomed flask fitted with a magnetic stirring bar and stopper. G-2-N (0.69 g, 50 mmol) was added and the solution was stirred for 30 min at room temperature. Solvent was evaporated and the resulting colourless oil, which solidified rapidly, was immediately recrystallized from heptane (30 ml) in a yield of 0.82 g (63%).

2-Phenyl-1,3,2-dioxaborolan-4-methanol nitrate. G-1-N was derivatized with phenylboronic acid, as described for G-2-N. After evaporation, the residual oil was extracted with 15 ml of heptane on a water-bath (70°C) and separated from the insoluble residue. The extract was concentrated under vacuum and gave a clear colourless oil (yield 1.1 g, corresponding to 85%), which crystallized very slowly. Recrystallization of the solid from heptane yielded a product with m.p. 39.5°C.

Spectroscopic characterization

Spectroscopic characterization with IR, NMR and MS was in agreement with the proposed structures.

Sample preparation

In a stoppered 20-ml centrifuge tube, 1 ml of plasma was spiked with 0.25 μg of the internal standard (50 μl of a 5 μg/ml solution of G-2-N in water) and agitated for a few seconds. Then 12 ml of diethyl ether, containing 0.06 ml of methanol, were added. Preliminary experiments demonstrated that this mixture of methanol and diethyl ether gave the optimal extraction yield. The tube was then shaken for 15 min and centrifuged at 2000 *g* for 5 min. The organic layer was removed with a pipette and transferred to a second centrifuge tube, containing 0.15 g of activated charcoal to reduce the background matrix. The mixture was then shaken for 5 min. After centrifugation (2000 *g*, 5 min) the solution was transferred to another tube and evaporated under nitrogen in a water-bath at 30°C. The residue was dissolved in 250 μl of a solution of phenylboronic acid in THF (50 mg/100 ml) and left to react for 5 min at room temperature.

The derivatization and extraction procedure for urine and venous tissue was the same as that for plasma. The veins were previously cut up into small pieces and taken up in 1 ml of water.

Faeces were diluted with an equal volume of water and then treated by the same procedure as urine.

Gas chromatography

Using a 5-μl SGE microsyringe, 1–2 μl of the THF solution were injected into the gas chromatograph. The carrier gas and scavenger gas was nitrogen at a flow-rate of 20 ml/min. The column oven temperature was 175°C and the injector block and detector temperatures were 230 and 240°C, respectively.

G-2-N was used as internal standard in the quantitation of G-1-N. The peak heights of the two nitrates on the chromatogram were measured. A calibration curve was constructed by measuring the ratio of the peak heights obtained by

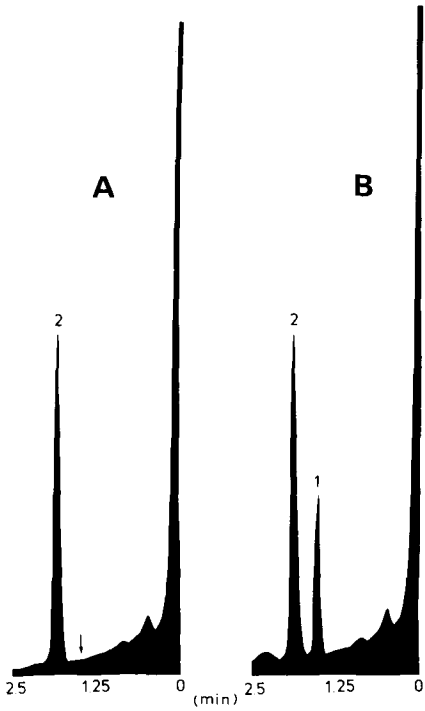

Fig. 1. Chromatograms of human serum: 1 ml of serum spiked with 600 ng of G-2-N (peak 2) derivatized with phenylboronic acid as described in Experimental. (A) Before administration of G-1-N; (B) sample obtained from a volunteer 0.2 h after oral administration of 20 mg of G-1-N. Peak 1 corresponds to 300 ng/ml G-1-N.

analysing plasma samples to which known amounts of G-1-N and G-2-N had been added.

RESULTS

Recovery

The recovery of G-1-N was estimated by comparing the peak heights obtained from the injection of known amounts of the derivatized compound with those obtained from the injection of extracts of plasma spiked with G-1-N. The results of the extraction recovery experiments are shown in Table I. Recovery from plasma without methanol in the extracting solvent was less than 65%. The overall mean recovery over all concentrations was 80.5%.

Precision studies

The within-day precision of the method was checked by analysing eight plasma samples spiked with different concentrations of G-1-N. Between-day precision was checked in four plasma samples every day for one week. The results are listed in Table II.

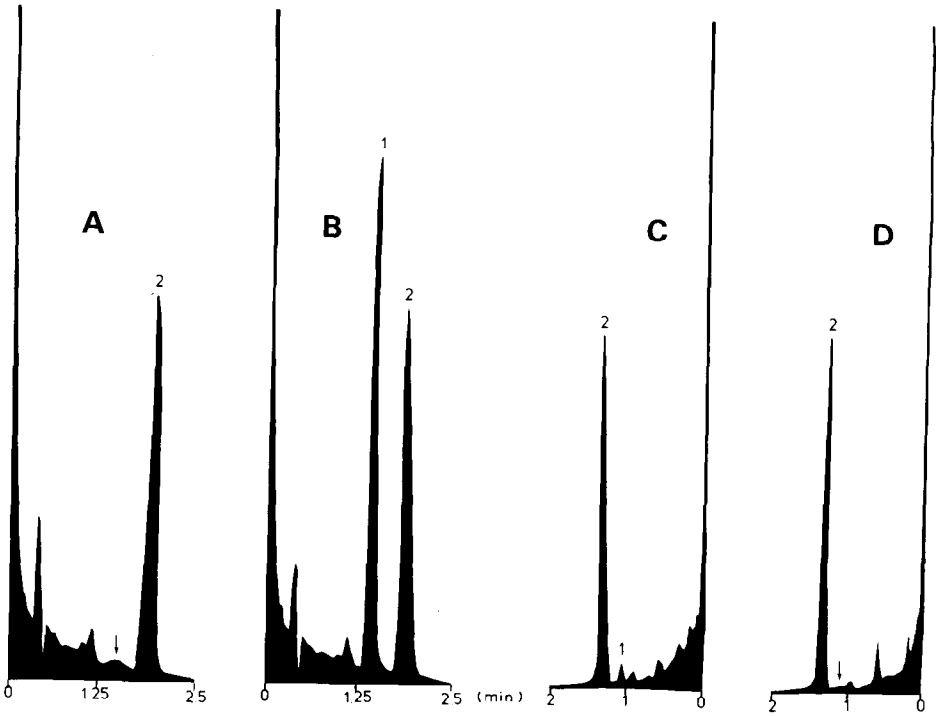

Fig. 2. (A) and (B) Chromatograms of canine urine: 1 ml of urine spiked with 1.20 μg of G-2-N (peak 2), derivatized with phenylboronic acid as described in Experimental. (A) Before administration of G-1-N; (B) fraction collected 12–24 h after administration of 2 mg/kg G-1-N (intravenously). Peak 1 corresponds to 1.55 μg/ml G-1-N. (C) and (D) Chromatograms of rat venous tissue: 0.02 g of tissue spiked with 300 ng of G-2-N (peak 2), extracted and derivatized with phenylboronic acid as described in Experimental. (C) Before administration of G-1-N; (D) fraction collected 10 h after administration of 32 mg/kg G-1-N intravenously. Peak 1 corresponds to 17 ng/g of G-1-N.

Linearity

The linearity of the method was confirmed for concentrations in the range 50–600 ng/ml; the correlation coefficients of the corresponding linear regressions ranged from 0.985 to 0.999.

Detection limit

The detection limit, defined at a signal-to-noise ratio of 3:1, was ca. 10 ng/ml G-1-N, corresponding to an absolute amount of 150 pg per injection.

TABLE I

EXTRACTION OF GLYCEROL 1-NITRATE FROM PLASMA ($n=9$)

Compound	Concentration added (ng/ml)	Recovery (mean ± S.D.) (%)
G-1-N	270	78.2 ± 5.2
	900	82.1 ± 4.6
	3000	81.3 ± 4.9
G-2-N	900	85.8 ± 3.9

TABLE II

REPRODUCIBILITY DATA FOR GLYCEROL 1-NITRATE IN PLASMA

Within-day data from eight plasma samples. Day-to-day data were from the same plasma samples on consecutive days. C.V. = coefficient of variation.

Concentration added (ng/ml)	Within-day ($n=8$)		Day-to-day ($n=4$)	
	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)
50	48.2 ± 2.43	5.0	46.9 ± 2.91	6.2
100	96.4 ± 5.01	5.2	98.3 ± 5.74	5.8
200	203.8 ± 9.94	4.9	190.2 ± 11.49	6.1
400	408.3 ± 16.78	4.1	423.6 ± 23.48	5.5
800	788.9 ± 35.47	4.5	771.1 ± 45.49	5.9

Accuracy

As a check of the reliability of the analytical results during routine application of the method, biological samples spiked with concentrations of G-1-N unknown to the analyst were examined. Table III presents the results of assays performed during a period of two years.

Studies with biological samples

A healthy volunteer received an oral dose of 20 mg of G-1-N in the form of water-soluble drops after an overnight fast. Fig. 1 shows chromatograms obtained from plasma samples taken before and after the administration. Fig. 4 represents the time course of the measured plasma concentrations. The terminal half-life of the concentrations measured was obtained by non-linear regression, according to a two-compartment model and using the computer program RIP [17] and came to 3.6 h.

In animal experiments, G-1-N was measured in canine urine after an intravenous dose of 2 mg/kg G-1-N and in various rat tissues after a 32 mg/kg bolus injection into the tail vein. Typical chromatograms from these experiments are reproduced in Fig. 2.

TABLE III

ASSAY OF GLYCEROL 1-NITRATE IN SPIKED BIOLOGICAL SAMPLES IN A BLIND STUDY

Sample	n	Range of given concentration (ng/ml or ng/g)	Difference of concentration given-found (% of given concentration)	
			Mean	Range
Human plasma	10	222-2678	5.6	−5.6 to +12.1
Human urine	4	254-944	2.6	−5.4 to + 4.4
Rat plasma	6	2500-51300	5.1	−7.4 to + 4.5
Rat tissue	6	1310-405600	4.5	−5.7 to + 7.0
Dog faeces	3	35-415	9.0	−7.5 to +15.3

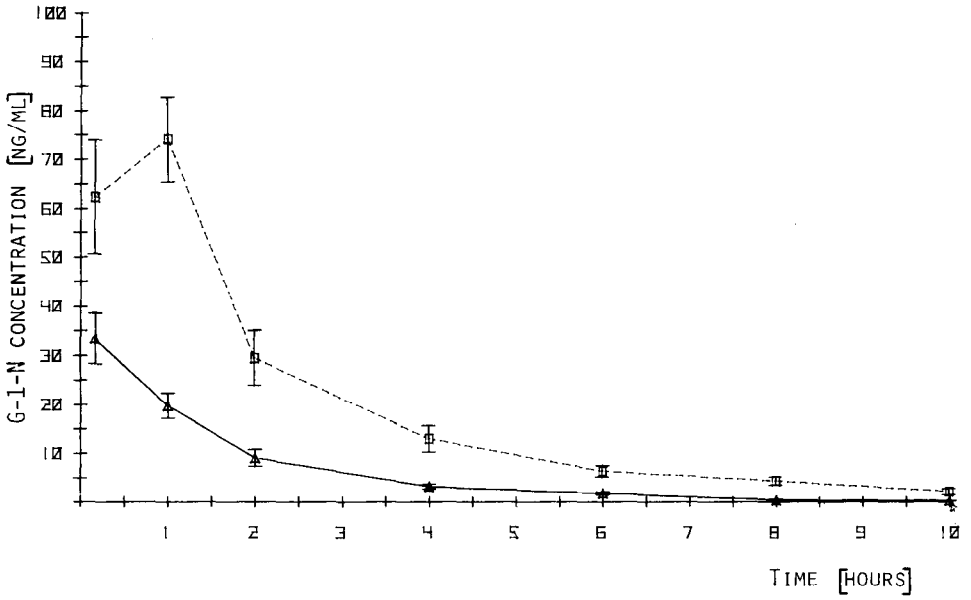

Fig. 3. Concentration (mean ± standard error of the mean) of G-1-N in the plasma (triangles) and in venous tissue (vena cava caudalis) (squares) of rats after administration of 32 mg/kg G-1-N as a bolus injection.

Fig. 3 shows the mean concentration–time curves in the plasma and in the venous tissue of the rat. For comparative purposes the denisty of the venous tissue was taken as 1 g/ml. The available amounts of venous tissue were of the order of

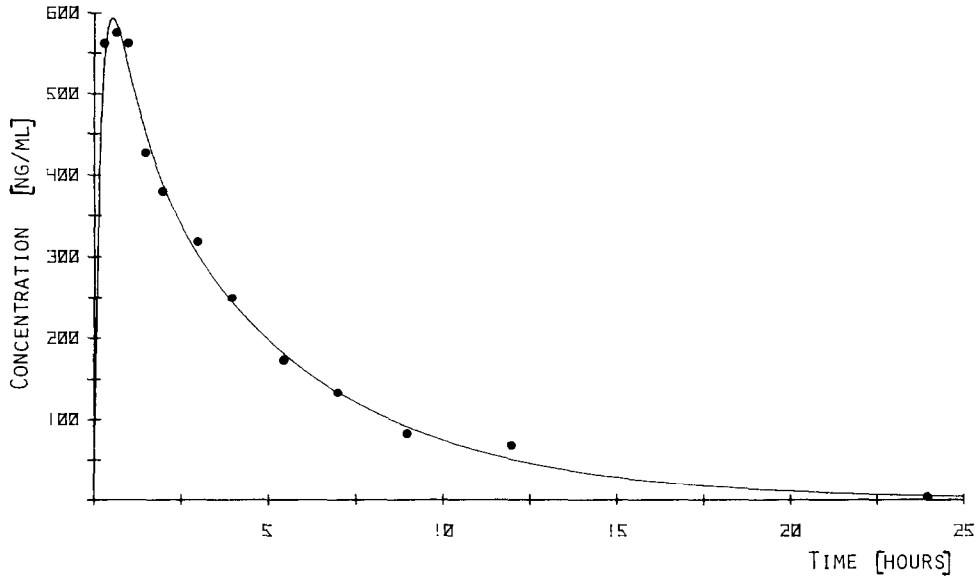

Fig. 4. Plasma concentration of G-1-N in a healthy volunteer after oral administration of 20 mg of G-1-N as drops. The calculation of the curve was based on the assumption of a two-compartment model.

20 mg (wet weight). The area under the tissue curve was ca. three times greater than the area under the plasma curve, indicating accumulation of G-1-N in the blood vessel wall.

DISCUSSION

It has been shown that derivatization with phenylboronic acid, with the formation of cyclic boronates, is a convenient way to convert the mononitrates of glycerol into suitable products for GC. Derivatization increases both the stability and the volatility of the mononitrates. A striking advantage of this procedure is that the reaction takes place very rapidly and with high yield. The time of the analysis is not increased by the derivatization, as no extra step is necessary: after evaporation of the extract the residues are taken up directly in the reagent mixture and the reaction is complete after standing for a few minutes at room temperature.

Underivatized G-1-N and G-2-N have very similar retention properties, both on packed and on capillary columns. This makes it difficult without derivatization to use the two isomers mutually as internal standards or to determine the two mononitrates simultaneously. On the other hand, the chemical similarity of the two mononitrates does speak for their use as mutual internal standards. For example, the lipophilicities of the two isomers are very similar: the *n*-octanol–water partition coefficients in the system *n*-octanol–water (phosphate buffer pH 7.4) are 0.26 (G-1-N) and 0.32 (G-2-N), which would lead to the prediction of similar extraction characteristics. These low partition coefficients might also suggest that the success of the extraction with diethyl ether–methanol (10:0.05) was simply the result of the use of relatively large volumes of extracting solvent. The derivatization with phenylboronic acid of G-1-N (a 1,2-diol) and G-2-N (a 1,3-diol) gives a five- and six-membered ring, respectively. The formation of these different-sized rings greatly facilitates the separation of the mononitrates.

Our experience with the analysis of G-1-N in biological material from various sources and of various sorts shows that the present procedure is insensitive to coextracted substances, even with a strongly contaminated matrix. For example, the extraction of faecal homogenates with diethyl ether usually gives voluminous viscous residues. Nevertheless, the analysis of G-1-N was not interfered with by peaks arising from the substances present in these residues.

In summary, it may be said that the robustness and speed of the present procedure make it particularly suitable for the routine analysis of glycerol mononitrates.

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