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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH SYNCHRONIZED ACCUMULATING RADIOISOTOPE DETECTOR TO ANALYSIS OF GLYCERYL TRINITRATE AND ITS METABOLITES IN RAT PLASMA

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SUMMARY

A new, sensitive, and specific high-performance liquid chromatographic method for the quantitative analysis of [^{14}C]glyceryl trinitrate and its four metabolites in plasma is described. The drugs are extracted from 0.05 ml of plasma with methanol and analyzed by high-performance liquid chromatography using a synchronized accumulating radioisotope detector. The limit of detection is 0.2 ng per injection. The within-day coefficient of variation is 5.9% at a concentration of 27.0 ng per ml of plasma. The method was applied to single-dose pharmacokinetics of glyceryl trinitrate in rat.

INTRODUCTION

Glyceryl trinitrate (GTN) has been used extensively for many years for the treatment of angina pectoris. It is known that GTN is rapidly metabolized by endogenous esterases to glyceryl dinitrates (GDNs) [1], which are about ten times less potent as vasodilators [2], and inactive mononitrates (GMNs) [3]. It is therefore essential to carry out simultaneous analysis of plasma levels of both GTN and its metabolites to perform pharmacokinetic studies of GTN in detail.

The analysis of GTN in plasma has been performed primarily by gas—liquid

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chromatography (GLC) with electron-capture detection [4—6]. Although this method possesses high sensitivity for GTN, it requires extensive clean-up procedures and solvent purification. Furthermore, GTN is thermally unstable and suffers decomposition on the GLC column [5]. It is a problem to achieve an acceptably accurate and reproducible analysis of GTN and its metabolites because the degradation product has a retention time equal to that of 1,2-glyceryl dinitrate (1,2-GDN).

Several high-performance liquid chromatographic (HPLC) methods have been reported for the analysis of GTN and its metabolites [7—10]. Although these methods can separate GTN from its mono- and dinitrate metabolites, the sensitivity and specificity are inadequate for biological samples due to use of spectrophotometric detection. Recently Spanggord and Keck [11] have reported a more sophisticated HPLC method with a thermal-energy analyzer to determine GTN and four degradation products. The disadvantages in this method are the costs of the thermal-energy analyzer detector used, and the lack of adequate accuracy.

Radioactive isotope (RI) tracer techniques have been widely applied to investigate the absorption, distribution, and biotransformation of GTN in animal species [12—14]. In this tracer technique thin-layer chromatography was used for the separation of GTN and its metabolites, but only partial resolution and low sensitivity were obtained. Baba et al. [15] have developed a synchronized accumulating radioisotope detector to detect radioactive substances eluted from an HPLC column. This radio-HPLC system gives satisfactory resolution with high detection efficiency.

The purpose of the present paper is to report a new radio-HPLC method for the analysis of GTN and its metabolites in rat plasma, and its application to the study of single-dose pharmacokinetics of GTN in rat.

MATERIALS AND METHODS

Chemicals and reagents

[U-^{14}C]Glyceryl trinitrate ([^{14}C]GTN) (224.6 μCi/mg) was supplied by Daiichi Kagaku Yakuhin (Tokyo, Japan). 1,2-Glyceryl dinitrate (1,2-GDN), 1,3-glyceryl dinitrate (1,3-GDN), 1-glyceryl mononitrate (1-GMN), and 2-glyceryl mononitrate (2-GMN) were synthesized in this laboratory. The methanol used for the mobile phase was of chromatographic purity and was obtained from Wako (Osaka, Japan). All other chemicals and solvents were analytical grade and were used without further purification.

Radio-HPLC system

The high-performance liquid chromatograph consisted of a Shimadzu (Kyoto, Japan) Model LC-2P solvent metering system, a Shimadzu UVD-2 ultraviolet (UV) detector (detection at 254 nm), an Aloka (Tokyo, Japan) synchronized accumulating radioisotope detector RLC-R17-748, and a Shimadzu two-pen recorder R-12.

A prepacked Chemcopak Zorbax-ODS (250 × 4.6 mm I.D.; particle size 8 μm; Chemco) was used. In order to avoid contamination of the analytical column, a pre-column (50 × 3.2 mm I.D.) tap-filled with Zorbax-ODS (particle

size 8 μm; Chemco) was placed between the injector and the analytical column. The mobile phase consisted of methanol—water (6:4, or 4:6). The mobile phase was degassed by ultrasonic vibration. The assays were performed at ambient temperature with a flow-rate of 1 ml/min. The elute from the HPLC column was mixed with a liquid scintillator* (7.0 ml/min) after UV detection and the resulting solution (8.0 ml/min) was passed through the five counting cells having an effective cell volume of 1.1 ml per cell.

Liquid scintillation counter

Authentic measurements of the radioactivity were conducted with the use of an Aloka LSC-502 liquid scintillation counter and corrections for quenching were achieved by using external standard. The sample was dissolved in 10 ml of scintillation solvent.

Sample preparation for RI-HPLC

To 0.05 ml of rat plasma sample was added 0.01 ml of 1 *M* silver nitrate and extracted three times with 0.2 ml of methanol. The organic layer was collected and condensed to about 0.1 ml. After centrifugation (1000 *g*, 10 min), 10—30 μl of the solution were subjected to RI-HPLC analysis.

Recovery

The absolute extraction recovery of [^{14}C]GTN from rat plasma was measured in the following way. To 0.05-ml portions of pooled blank rat plasma were added 5 nCi of [^{14}C]GTN dissolved in 10 μl of saline. The sample was extracted and concentrated according to the sample preparation procedure. The concentrated sample was subjected to liquid scintillation counting. Absolute extraction recovery was calculated by comparing the radioactivity from the [^{14}C]GTN added with the radioactivity from the plasma extract. The absolute extraction recovery of metabolites was estimated as follows. The accurate disintegration counts in the rat plasma (0.05 ml each) at 8 and 15 min after intravenous administration of [^{14}C]GTN (20 μCi) were made by a liquid scintillation counting method. The same rat plasma (0.05 ml each) was extracted and concentrated according to the sample preparation for RI-HPLC. The concentrated extracts were subjected to liquid scintillation counting. Absolute extraction recovery was calculated by comparing the radioactivity in the plasma with the radioactivity in the plasma extract.

Detection efficiency

The detection efficiency of this RI-HPLC system for [^{14}C]GTN was estimated by comparing the counts under the peak obtained from the injection of known amounts of [^{14}C]GTN with the counts of [^{14}C]GTN measured by a liquid scintillation counter. The detection efficiency for [^{14}C]GTN metabolites was estimated as follows. An accurate disintegration count in the rat plasma sample at 5 min after intravenous administration of [^{14}C]GTN was made by a liquid scintillation counting method. The rat plasma sample was

*Dioxane—toluene—ethyl cellosolve (75:15:10, v/v) containing 100 g of naphthalene, 4 g of 2,5-diphenyloxazole, and 0.4 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per litre.

then injected into the RI-HPLC system and the total counts under the peaks were calculated. The detection efficiency was estimated by comparing the total counts with the injected accurate counts.

Reproducibility

A male rat was injected intravenously with a single dose of 20 μCi of [^{14}C]GTN. After 5 min the rat was sacrificed and 8 ml of blood were collected. Aliquots (0.5-ml) of the resulting plasma were processed as described by the sample preparation for RI-HPLC. The methanolic preparations of 10 μl of the concentrated sample were injected into the RI-HPLC four times. In addition, four aliquots (0.3 ml each) of the same rat plasma sample were analyzed in replicate according to the sample preparation and RI-HPLC procedures described above.

Pharmacokinetics of a single intravenous dose of [^{14}C] GTN in rat

Three male Wistar-SPF albino rats, weighing approximately 300 g, were injected intravenously into the right jugular vein with a single dose of [^{14}C]GTN (112.3 μCi/kg) dissolved in 0.3 ml of saline. Heparinized blood samples (0.15—0.2 ml) were taken from the left jugular vein at 2, 5, 10, 20, 30, 60, and 120 min after injection. The plasma was obtained by centrifuging the blood at 1000 *g* for 10 min. The plasma samples were divided into two sets. Each plasma sample in one set (0.02 ml) was subjected to liquid scintillation counting and the total radioactivity in the plasma was determined. Each plasma sample in the other set (0.05 ml) was processed as described by the sample preparation for RI-HPLC and then subjected to RI-HPLC analysis. The estimation of the amounts of [^{14}C]GTN and its metabolites in plasma was based on the relative peak intensities on the chromatogram and the total radioactivity measured by a liquid scintillation counter.

RESULTS AND DISCUSSION

It is well known that GTN is rapidly degraded in rat plasma with a half-life of about 20 min at 37°C [16]. Yap et al. [4] reported that this degradation of GTN could be inhibited for at least 1.5 h by the addition of silver nitrate in a 0.05 *M* final concentration. It was decided to add silver nitrate to the rat plasma sample.

The absolute extraction recovery of [^{14}C]GTN from spiked rat plasma was 86.5 ± 0.5% (n = 4). The absolute extraction recovery of total ^{14}C-radioactivity from drug-supplemented rat plasma was 84.7% (n = 2) for the plasma taken 8 min (GTN, GDNs, GMNs), and 81.0% (n = 2) for the plasma taken 15 min (GDNs, GMNs) after administration of [^{14}C]GTN. These data seem to suggest that methanol is an efficient extraction solvent for [^{14}C]GTN and its metabolites ([^{14}C]GDNs, [^{14}C]GMNs) and there is no difference between [^{14}C]GTN and its metabolites in extraction efficiency.

The mobile phases studied included various ratios of methanol—water, which is a simple and commonly used solvent combination for reversed-phase chromatography. The resolution could be regulated by changing the methanol concentration. When the methanol concentration is 60%, the retention times for

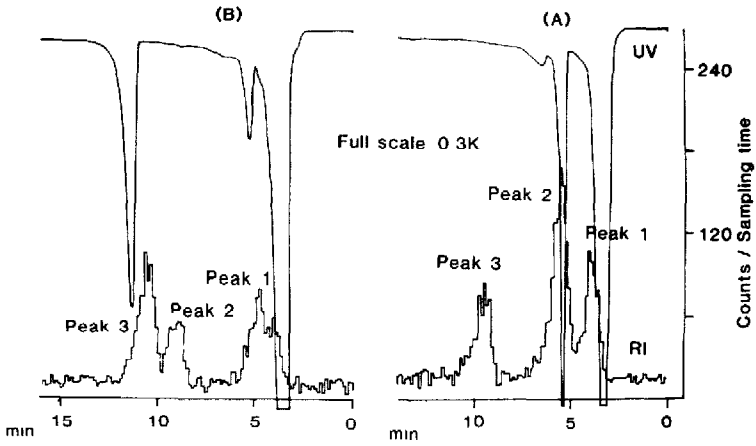

Fig. 1. Radioisotope high-performance liquid chromatograms for an extract of a plasma sample 2 min after intravenous administration of [^{14}C]GTN (112.3 μCi/kg). (A) Mobile phase = 60% methanol; peak 1 = GMNs, Peak 2 = GDNs, peak 3 = GTN. (B) Mobile phase = 40% methanol; peak 1 = GMNs, peak 2 = 1,3-GDN, peak 3 = 1,2-GDN.

GTN, a mixture of GDNs, and a mixture of GMNs are 9.5, 5.4, and 3.7 min, respectively. Using 40% methanol, 1,2 and 1,3-glyceryl dinitrate can be separated with retention times of 10.7 and 9.0 min, respectively. In view of these results 60% and 40% of methanol in water were employed in the analysis. Fig. 1 shows chromatograms of a plasma sample which was collected from a rat at 2 min after intravenous administration of [^{14}C]GTN [methanol 60% (A), 40% (B)]. The retention time of each peak was compared with that of a reference non-labeled compound. The t_R values of peaks 1, 2 and 3 in Fig. 1A were similar to those of GMNs, GDMs and GTN, respectively. The t_R values of peaks 1, 2 and 3 in Fig. 1B were similar to those of reference GMNs, 1,3-GDN and 1,2-GDN, respectively. When a UV detector was used for HPLC effluents, many peaks were found which did not originate from the drug. When the RI detector is used, therefore it is important to clarify in advance which peaks in the HPLC are being referred to.

The detection efficiencies of the RI-HPLC system for authentic [^{14}C]GTN and its metabolites in rat plasma sample were 92.3 ± 2.5 (n = 3) and 89.1 ± 0.9% (n = 4), respectively. The results demonstrate excellent detection efficiencies not only for [^{14}C]GTN but also for its metabolites, and suggest that there is no difference between [^{14}C]GTN and its metabolites in detection efficiency.

The reproducibility in counting of the RI-HPLC was determined by injecting the same test rat plasma sample into the RI-HPLC system four times. The results are shown in Table I. The present method obviously provides very good reproducibility of counting. The within-day precision of the RI-HPLC analysis for [^{14}C]GTN and its metabolites was good, as assessed by conducting replicate (n = 4) analyses of the same rat plasma at 5 min after intravenous administration of [^{14}C]GTN (Table II). It is apparent that the present method has a better reproducibility than the thermal-energy analyzer method (GTN 0.5 ng, C.V. 25%; 13.2 ng, C.V. 12%) [11].

TABLE I

REPRODUCIBILITY IN COUNTING

	Counts		
	GMNs	GDNs	GTN
1	990	1940	329
2	996	1916	372
3	1030	1911	343
4	1046	1952	336
Mean	1016	1930	345
S.D.	26.9	19.5	18.9
C.V. (%)	2.6	1.0	5.5

TABLE II

REPRODUCIBILITY OF ANALYSES OF [^{14}C]GTN AND ITS METABOLITES IN RAT PLASMA

	ng/ml plasma		
	GMNs	GDNs	GTN
1	42.7	118.7	27.3
2	43.7	115.7	27.7
3	42.3	116.7	28.3
4	41.7	116.3	24.7
Mean	42.6	116 9	27.0
S.D.	0 84	1.30	1.59
C.V. (%)	2.0	1.1	5.9

In the RI-HPLC method the detection limit was reported as about 45 pCi for ^{14}C [15]. The lower limit of detection for [^{14}C]GTN was found to be about 0.2 ng injected, since the specific activity of [^{14}C]GTN was 224.6 μCi/mg.

The time course of plasma concentrations of [^{14}C]GTN and its metabolites following intravenous administration of [^{14}C]GTN in three rats is shown in Fig. 2. The half-lives of [^{14}C]GTN and its metabolites are presented in Table III. The data give some insight into interindividual differences. It should be pointed out that the ability to use RI-HPLC which gives satisfactory resolution with high detection efficiency is a great advantage to evaluate interindividual differences of pharmacokinetics in small laboratory animals. The disappearance of [^{14}C]GTN in plasma followed first-order kinetics and was very rapid. By 20 min there was no detectable unchanged [^{14}C]GTN. The elimination half-life of [^{14}C]GTN was 2.2 ± 0.21 min. This value agrees with the values found using a GLC method [17]. The appearance of 1,3-[^{14}C]GDN and 1,2-[^{14}C]GDN was very rapid. In the first blood sample taken 2 min after the administration of [^{14}C]GTN, peak concentration of GDNs was detected.

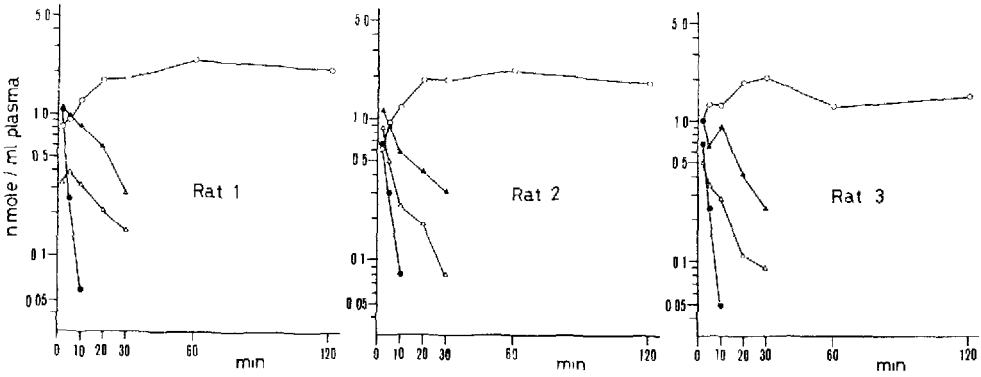

Fig. 2. Plasma concentrations of GTN (•), 1,2-GDN (▲), 1,3-GDN (△), and GMNs (○) as a function of time after intravenous administration of [^{14}C]GTN (112.3 μCi/kg) in male rats.

TABLE III

PLASMA HALF-LIVES OF [^{14}C]GTN AND ITS METABOLITES IN PLASMA AFTER INTRAVENOUS ADMINISTRATION OF [^{14}C]GTN IN MALE RATS

	Min		
	1,2-GDN	1,3-GDN	GTN
1	14.6	21.2	1.9
2	15.3	9.1	2.6
3	14.3	11.1	2.1
Mean	14.7	13.8	2.2
S.E.	0.29	3.75	0.21

Thereafter a monoexponential decline in the plasma levels was observed, and 30 min after [^{14}C]GTN administration the levels were below the limit of detection (0.05 nmol per ml of plasma). Plasma [^{14}C]GMNs levels increased gradually after the administration of [^{14}C]GTN. Peak plasma [^{14}C]GMNs concentrations were generally detected between 30 and 60 min after [^{14}C]GTN administration. The rate of disappearance of [^{14}C]GMNs was slower than of [^{14}C]GTN and [^{14}C]GDNs.

The present synchronized accumulating radioisotope detector HPLC method allows the simple, sensitive, rapid, and selective determination of [^{14}C]GTN and its metabolites simultaneously in rat plasma with good accuracy and precision. The method has made it possible to perform pharmacokinetic studies of GTN and to investigate interindividual variations in small laboratory animals.

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