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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC QUANTITATION OF TRIACYLGLYCEROLS CONTAINING FENOPROFEN FROM BIOLOGICAL SAMPLES

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SUMMARY

A normal-phase high-performance liquid chromatographic (HPLC) method has been developed for the quantitation of radiolabelled triacylglycerols containing fenoprofen, synthesized from [^{3}H]glycerol by isolated hepatocytes and adipocytes. The assay consists of extracting the lipids into diethyl ether, separating triacylglycerols from polar endogenous lipids using silica Sep-Pak^{TM} cartridges and quantitating endogenous triacylglycerols and triacylglycerols containing fenoprofen by HPLC resolution and scintillation counting. HPLC separation is achieved in less than 10 min. Using [^{14}C]tripalmitin as internal standard the assay has a linear relationship between added triacylglycerol and measured endogenous triacylglycerols and triacylglycerols containing fenoprofen with regression coefficients of 0.997 and 0.998, respectively.

INTRODUCTION

Xenobiotic carboxylic acids can substitute for endogenous fatty acids and become esterified with glycerol to form hybrid triacylglycerols [1]. These xenobiotic lipids may form long-lived residues in tissues and have pharmacological and toxicological consequences in their own right or via the release of stored acid [2].

Various 2-arylpropionate non-steroidal anti-inflammatory drugs are incorporated into hybrid triacylglycerols [1]. In order to study their formation a sensitive analytical method, capable of resolving hybrid triacylglycerols from endogenous triacylglycerols, is required. Thin-layer chromatography (TLC) has

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been employed to detect the presence of these xenobiotic triacylglycerols [1, 3], however, this is a laborious and time-consuming method. Recently a normal-phase high-performance liquid chromatographic (HPLC) technique with low-wavelength UV detection was developed for the quantitative separation of neutral lipids [4]. For our purposes this method has been modified in order to resolve endogenous and hybrid triacylglycerols as two separate lipid classes. Radiometric detection has been employed to overcome the lack of sensitivity of UV detection and this allows quantitation of these compounds from isolated hepatocytes and adipocytes.

EXPERIMENTAL

Synthesis of triacylglycerols containing fenoprofen

1,2-Dipalmitoyl-3-[2-(3-phenoxyphenyl)propanoyl]-glycerol was prepared from 1,2-dipalmitoyl-*sn*-glycerol (Sigma, St. Louis, MO, U.S.A.) and racemic fenoprofen (Lilly Industries, Sydney, Australia) via the acid chloride, using the general approach described by Crayford and Hutson [5] for similar xenobiotic-containing triacylglycerols. Fenoprofen (42.6 mg, 0.1 m*M*) in 2 ml of dichloromethane and 0.2 ml of thionyl chloride was refluxed for 2 h, and the solvent and excess thionyl chloride were removed with a stream of dry nitrogen. The residue was dissolved in 1 ml of dry benzene and evaporated to dryness; this process was repeated.

The resulting crude fenoprofen acid chloride was mixed with 2 ml of dichloromethane containing 0.05 ml of dry pyridine and 114 mg (0.2 m*M*) 1,2-dipalmitoyl-*sn*-glycerol, and allowed to stand at room temperature for 24 h. The solvent was removed with dry nitrogen, the residue redissolved in 2 ml of dichloromethane, loaded onto a 20×1 cm chromatographic column containing Kieselgel 60, 70–230 mesh (E. Merck, Darmstadt, F.R.G.) and eluted with dichloromethane. The column eluent was collected in 5-ml fractions and each was examined for product using a 20 cm long TLC plate of Kieselgel 60 F_{254} (E. Merck) developed with hexane–diethyl ether–glacial acetic acid (50:50:1). Plates were examined with UV light (254 nm) and at 360 nm after spraying with 7-chlorofluoresceine. Fractions 19–21 contained material with R_F 0.66, which both absorbed light at 254 nm and fluoresced at 360 nm after treatment with 7-chlorofluoresceine. These fractions were evaporated to yield 52 mg of a white solid which was further characterised by HPLC and mass spectrometry (MS). Mass spectra were obtained by direct insertion using an MS30 mass spectrometer operating at 70 eV. Perfluorokerosene was used as reference in the second beam.

Preparation of standard [^{3}H] triacylglycerols

Isolated hepatocytes were prepared from male Hooded Wistar rats by the method of Seglen [6] and incubated for 1 h at 37°C in Waymouth's medium in the presence of carbon dioxide–oxygen (5:95). Each incubation contained $2 \cdot 10^6$ viable hepatocytes, 1 m*M* racemic fenoprofen and 10 μCi of [1(3)^{3}H]glycerol (Amersham). Material from fifteen incubations was pooled and stored at −20°C,

and was subsequently used to calibrate the method. A series of control incubations were also carried out in the absence of fenoprofen.

Characterisation of [^{3}H] triacylglycerols containing fenoprofen

The characterisation of the [^{3}H]triacylglycerols containing fenoprofen, prepared from rat isolated hepatocytes, was determined by co-chromatography with synthetic standards using both TLC and HPLC. Hepatocyte extracts were prepared as described under *Sample preparation* but without the use of silica precolumns (Sep-Pak) and applied to 20×5 cm silica gel 60 F_{254} plates (E. Merck) as previously described. The ^{3}H activity on each plate was quantitated by counting 1-cm strips along the length of each plate and the R_F of any measured ^{3}H activity was compared with synthetic standards also spotted onto each plate. HPLC analysis was carried out as decribed in *Normal-phase HPLC.*

Sample preparation

Incubation mixture (0.5 ml) consisting of $0.5 \cdot 10^6$ hepatocytes in Waymouth's medium were placed in a 10-ml glass culture tube fitted with a PTFE-lined screw cap. Each tube contained diethyl ether (3 ml) and $5 \cdot 10^3$ dpm of [^{14}C]tripalmitin as internal standard (Amersham). The contents of each tube were mixed on a rotary mixer for 10 min and the layers separated by centrifugation for 2 min at 1500 *g*. The diethyl ether layer was placed in a 5-ml disposable glass tube and evaporated under reduced pressure (Speed Vac concentrator, Servant Instruments, Hicksville, NJ, U.S.A.). The residue was redissolved in 0.2 ml of eluting solvent consisting of diethyl ether–hexane–glacial acetic acid (33:66:0.1). Each sample was loaded onto a 2.5 cm×1.0 cm I.D. silica column (Sep-Pak, Millipore-Waters, Milford, MA, U.S.A.) and eluted with 14 ml of eluting solvent, with the aid of positive pressure applied with a glass syrine. The first 4 ml of solvent, which contained endogenous [^{3}H]triacylglycerols (endogenous TAG), [^{14}C]tripalmitin and [^{3}H]triacylglycerols containing fenoprofen (fenoprofen-TAG), was collected into a 5-ml glass tube and evaporated to dryness under reduced pressure as described above.

Samples were dissolved in 0.1 ml of hexane and the whole volume injected into the HPLC system.

Normal-phase HPLC

Chromatography was carried out using a 250 mm×40 mm I.D. silica column (5-μm LiChrosorb, E. Merck). The mobile phase was hexane–isopropanol–glacial acetic acid (100:0.25:0.1) and had a flow-rate of 2.0 ml/min, maintained with a pump (Beckman Model 114M) at a back-pressure of approximately 10 300 MPa (100 bar). Peaks were initially detected for synthetic standard fenoprofen-TAG and tripalmitin using a variable-wavelength UV detector (Millipore-Waters Model 481) at 211 nm. ^{3}H or ^{14}C peaks were identified by collecting 30-s samples of column effluent, and the radioactivity was measured using scintillant (Beckman, Ready Solv EP) and a scintillation counter with a dual-label capability (Beckman, Model LS 3801). Retention windows were set prior to each chromatographic run using both [^{14}C]tripalmitin and the standard

[^{3}H]triacylglycerol mixture which contained both fenoprofen-[^{3}H]TAG and endogenous [^{3}H]TAG.

Calibration

The method was calibrated by making serial dilutions of the standard [^{3}H]triacylglycerol hepatocyte preparation to produce five samples in the range of $2 \cdot 10^3$ to $1 \cdot 10^5$ dpm of total ^{3}H activity. Standard samples were taken through the analytical method, and activity corresponding to fenoprofen-[^{3}H]TAG and both endogenous [^{14}C]- and [^{3}H]-TAG was determined in the appropriate fraction.

For each sample, ^{3}H recovered in the endogenous triacylglycerol fraction and in the fenoprofen-TAG fraction was divided by the ^{14}C internal standard activity recovered and these ratios were plotted against the dilution of the standard hepatocyte preparation, in order to evaluate the linearity and working range of the method. An estimate of the reproducibility of the method was obtained by dividing the measured ^{3}H/^{14}C ratio by the dilution to give a normalised recovery from which a mean and coefficient of variation were calculated for each calibration curve.

In the routine application of the method, which involved the analysis of forty samples chromatographed over a period of approximately 8 h, an incubation prepared for quality-control purposes was split into four samples each containing approximately $4 \cdot 10^3$ dpm of ^{3}H. These samples were chromatographed at evenly spread intervals throughout the set of unknown samples, and the ratio of ^{3}H fenoprofen-TAG or endogenous TAG activity divided by ^{14}C internal standard activity was calculated. A mean ^{3}H/^{14}C ratio and coefficient of variation were determined for both endogenous and fenoprofen-containing triacylglycerols.

Quantitation of fenoprofen-TAG activity in an unknown sample

To each sample a known quantity of [^{14}C]tripalmitin (internal standard) was added prior to extraction. Samples were then processed and chromatographed as described, and the ^{3}H and ^{14}C activity in the endogenous TAG and fenoprofen-TAG fractions was measured.

The ^{3}H fenoprofen-TAG activity in an unknown sample (fenoprofen-TAG) was calculated as follows:

$$\text{fenoprofen-TAG} = \frac{\text{fenoprofen-TAG}_{\text{obs}} \times \text{I.S.}}{\text{I.S.}_{\text{obs}}} \qquad (1)$$

where fenoprofen-TAG$_{\text{obs}}$ is ^{3}H activity recovered in the fenoprofen-TAG fraction, I.S.$_{\text{obs}}$ is ^{14}C activity recovered in the endogenous TAG fraction, and I.S. is ^{14}C activity added to the sample prior to extraction.

Quantitation of fenoprofen-TAG activity by this method relies on the assumption that there is equal extraction of [^{14}C]tripalmitin and fenoprofen-[^{3}H]TAG during sample preparation. In order to confirm that this was the case, pure fenoprofen-[^{3}H]TAG was prepared from the hepatocyte extracts using HPLC as described in the section *Normal-phase HPLC*. From this four hepatocyte standards containing known ^{3}H activities of fenoprofen-TAG in the range 400–8000

dpm were prepared and analysed in duplicate. The fenoprofen-TAG activities were calculated using eqn. 1. Regression analysis of fenoprofen-TAG activity added against fenoprofen-TAG activity calculated was carried out in order to establish the validity of the method.

Application

The method has recently been applied to enzyme kinetic studies of the synthesis of triacylglycerols containing fenoprofen, from fenoprofen and [^{3}H]glycerol in both isolated hepatocytes and adipocytes. Isolated adipocytes were prepared in Krebs–Henseleit buffer by the method of Rodbell [7] and incubations were similar to those described for hepatocytes except that $1 \cdot 10^5$ cells in 1 ml of buffer were incubated with 50 μCi of [^{3}H]glycerol and varying concentrations of fenoprofen. Aliquots of 0.5 ml were assayed as described for the hepatocyte incubations.

RESULTS AND DISCUSSION

Characterisation of synthetic fenoprofen-TAG

The base peak was at *m/z* 197. Peaks at 224 and 242 are likewise characteristic of the aromatic acid. Peaks at 256 and 239 are characteristic of palmitic acid [8]. Other significant peaks were found at *m/z* 536, 537, 538, 549, 550, 551, 554, 555, 595 and 596. The peak at 537 (19%) was of greater intensity than 536 (9%), therefore the fragmentation $(M-RCO_2)^+$ is more important than $(M-RCO_2H)^+$ and the aromatic acid is directing the fragmentation of palmitate. The similar fragmentation of the aromatic acid was not favoured, and peaks in the region of 550 were of low intensity. The most puzzling feature was the appearance of a major ion at *m/z* 554 (16%). This could indicate a monopalmitoyl monoaromatic acid diglyceride. The alternative conclusion is that an unusual rearrangement of a palmitoyl group must have occurred involving a ketene loss. The mass spectrum is consistent with the structure of 1,2-dipalmitoyl-3-[2-(3-phenoxyphenyl)propanoyl]glycerol.

Characterisation of [^{3}H] lipids

The results of TLC analysis of the [^{3}H] lipids formed by isolated rat hepatocytes are shown in Table I. A portion of the ^{3}H activity detected corresponded to diacylglycerols, monoacylglycerols, phospholipids and glycerol, however, the majority of ^{3}H activity was divided into two distinct areas, one co-chromatographing with synthetic tripalmitin and the other with synthetic fenoprofen-TAG. The activity corresponding to synthetic fenoprofen-TAG was only present in extracts of hepatocytes incubated in the presence of fenoprofen, and only background activity was detectable with boiled hepatocytes, indicating that its presence is due to enzymatic synthesis only in viable hepatocytes.

The results of HPLC analysis (Fig. 1) show the same pattern as that observed with TLC analysis (Table I), in that ^{3}H activity corresponding to fenoprofen-TAG was present only in incubations containing fenoprofen, and co-chromatographed with the synthetic fenoprofen-TAG 1,2-dipalmitoyl-3-[2-(3-phenoxyphenyl)propanoyl]glycerol.

TABLE I

THIN-LAYER CHROMATOGRAPHY OF [^{3}H]LIPID EXTRACTS

Synthetic standard	R_F value	dpm recovered		
		Boiled hepatocytes	Viable hepatocytes	
		+Fenoprofen*	−Fenoprofen*	+Fenoprofen*
Tripalmitin	0.77	−**	33866	25497
Fenoprofen-TAG	0.66	−	121	17401
Dipalmitin	0.34	−	5480	3169
Monopalmitin, phospholipid, glycerol	0–0.06	8500	9926	9716

*Racemic fenoprofen 1 m*M*.
**Background (70–150 dpm).

Taken together these data indicate that the ^{3}H-labelled material co-chromatographing with synthetic fenoprofen-TAG is fenoprofen-TAG synthesised only by viable hepatocytes and it is not a normal endogenous lipid.

Selectivity of sample preparation

During the initial development of the HPLC conditions it was observed that the retention times for diacylglycerols, monoacylglycerols, phospholipids and

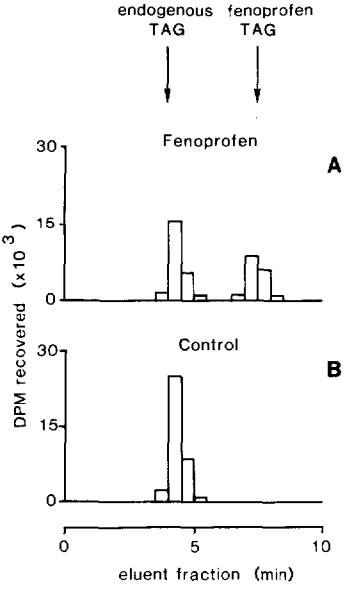

Fig. 1. Radiochromatograms of triacylglycerols extracted from incubations of isolated rat hepatocytes carried out in the presence of (A) 10 μCi [^{3}H]glycerol and 1 m*M* fenoprofen and (B) 10 μCi [^{3}H]glycerol only. The arrows indicate the UV retention times of tripalmitin and synthetic 1,2-dipalmitoyl-3-[2-(3-phenoxyphenyl)propanoyl]glycerol (fenoprofen-TAG).

TABLE II

REGRESSION ANALYSIS OF FOUR CALIBRATION CURVES

All values expressed as mean ± S.E.M.

Lipid	Regression analysis ($y=a+bx$)		
	a	b	r^2
Endogenous TAG	0.241 ± 0.078	15.294 ± 0.735	0.997 ± 0.001
Fenoprofen-TAG	0.120 ± 0.062	11.502 ± 0.254	0.998 ± 0.001

glycerol were greater than 1 h. As demonstrated by TLC analysis of the hepatocyte extracts (Table I), in addition to triacylglycerols, other classes of lipids were also synthesised from [^{3}H]glycerol and extracted. The presence of this slowly eluting radioactivity results in potential interference during HPLC quantitation. Attempts were made to selectively extract endogenous TAG and fenoprofen-TAG with a variety of solvents without success. Diethyl ether was finally chosen as the extracting solvent since it extracts greater than 90% of [^{14}C]tripalmitin but less than 0.01% of [^{3}H]glycerol from spiked blank hepatocytes.

In order to separate triacylglycerols from other endogenous lipids, the method of Hamilton and Comai [4] employing silica precolumns (Sep-Pak) was modified. The efficacy of Sep-Pak separation was assessed using TLC under the previously described conditions. TLC analysis of samples processed with the silica cartridges showed that ^{3}H activity was present only in the areas corresponding to fenoprofen-TAG and endogenous TAG. The ratio of endogenous TAG/fenoprofen-TAG was the same, regardless of whether or not a silica cartridge was used, indicating that there was no selective loss of either endogenous TAG or fenoprofen-TAG due to the clean-up procedure.

Normal-phase HPLC

The HPLC retention times obtained using UV detection for tripalmitin, synthetic fenoprofen-TAG and fenoprofen are 4.00, 7.08 and 20.80 min, respectively. The resolution of [^{14}C]tripalmitin and [^{3}H]triacylglycerols as determined by counting 0.5-min fractions of eluent is shown in Fig. 1. The two classes of triacylglycerols are well resolved and the labelled materials co-chromatograph with their corresponding synthetic standards.

Calibration and reproducibility

The mean data of four serial dilution calibration curves are shown in Table II. There is a linear relationship between ^{3}H activity added and both ^{3}H endogenous TAG/^{14}C internal standard activity and ^{3}H fenoprofen-TAG/^{14}C internal standard activity recovered, with the mean (± standard error of the mean, S.E.M.) ^{3}H/^{14}C ratios recovered for endogenous TAG and fenoprofen-TAG being 17.13 ± 0.64 and 12.19 ± 0.44, respectively. The corresponding mean (± S.E.M.) coefficients of variation for endogenous TAG and fenoprofen-TAG were 8.9 ± 1.6 and 6.4 ± 1.8%, respectively. There is, therefore, good reproducibility both within

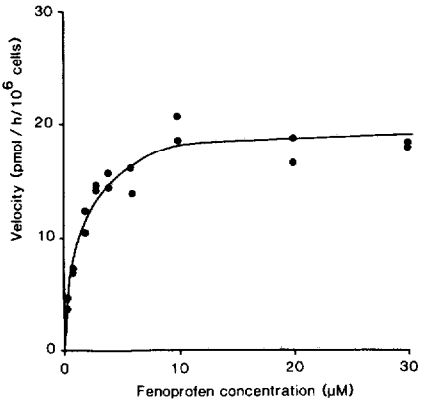

Fig. 2. Synthesis of fenoprofen-TAG by isolated rat adipocytes incubated in the presence of racemic fenoprofen and 50 μCi [^{3}H]glycerol.

a calibration curve run on a particular day and between calibration curves run on different days.

In the routine application of the method collection of the appropriate triacylglycerol eluent fractions relied completely on retention windows set at the start of each run and which showed minor variations from day to day. In order to determine whether chromatographic conditions were changing during each analytical period a series of four quality-control samples were evenly spread throughout the set of unknown samples. Because of the dependence of the method on accurate collection of samples in the retention windows, we recommend that quality-control samples such as those described be routinely used in this analysis. Six sets of quality-control samples were analysed. The mean ($\pm$S.E.M.) coefficients of variation of the ^{3}H/^{14}C ratios for endogenous TAG and fenoprofen-TAG were 4.2 ± 0.6 and $3.4 \pm 0.7\%$, respectively, indicating that chromatographic conditions remained stable over approximately 8 h of each analytical run, and therefore there were no systematic errors due to changes in retention times.

The ability of the assay to quantitate fenoprofen-TAG activity in unknown samples using eqn. 1 was assessed by regression analysis of a calibration curve containing known quantities of chromatographically pure fenoprofen-[^{3}H]TAG. The following expression was obtained with an r^2 value of 0.999: fenoprofen-TAG $= 1.159$ fenoprofen-TAG$_{init} - 127.3$ where fenoprofen-TAG is the calculated activity and fenoprofen-TAG$_{init}$ is the known ^{3}H activity added to the sample prior to extraction. Statistical analysis [9] of this result shows that the slope is not significantly different from 1 ($p > 0.05$) and that the intercept is not significantly different from 0. This result supports the underlying assumption in eqn. 1.

Application

Adipose tissue is one of the major sites of triacylglycerol synthesis and storage, and this analytical method was applied to measure the formation of fenoprofen-TAG by fenoprofen in rat isolated adipocytes. As shown in Fig. 2, there is saturable synthesis of fenoprofen-TAG consistent with Michaelis–Menten kinetics

and in this example the K_M of 1.5 μM is within the range of plasma concentrations of unbound fenoprofen normally achieved clinically.

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