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THE ANALYSIS OF [^{14}C]CLOFIBRIC ACID GLUCURONIDE AND [^{14}C]CLOFIBRIC ACID IN PLASMA AND URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

P.J. MEFFIN* and D.M. ZILM

Department of Clinical Pharmacology, The Flinders University of South Australia, Bedford Park, 5042 South Australia (Australia)

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SUMMARY

A high-performance liquid chromatographic method has been developed for the quantitation of [^{14}C]clofibric acid glucuronide and [^{14}C]clofibric acid using conventional scintillation counting. The assay has a linear relationship between the added and observed ratios of clofibric acid glucuronide : clofibric acid in the range of 0.001—0.6 for plasma and 0.5—100 for urine, and is able to quantitate previously unmeasurable concentrations of clofibric acid glucuronide in plasma.

INTRODUCTION

We have previously reported a high-performance liquid chromatographic (HPLC) method for clofibric acid glucuronide (CFAG) which does not require hydrolysis to clofibric acid (CFA) [1]. This method, which uses the ultraviolet (UV) absorbance of CFAG as the means of detection, is insufficiently sensitive to measure plasma concentrations of CFAG in man [2]. In order to overcome this difficulty the previously described chromatographic method has been modified to allow detection of [^{14}C]CFAG and [^{14}C]CFA with scintillation counting which results in greatly increased sensitivity. The method has been applied to an animal model of CFA disposition.

EXPERIMENTAL

Biosynthesis and purification of [^{14}C]clofibric acid glucuronide

The methods used for the preparation and purification of [^{14}C]CFAG ([^{14}C]clofibric acid glucuronide) are a modification of those previously

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described for the preparation of unlabelled CFAG [1]. Each of two male hooded Wistar rats received 925 kBq of [1-^{14}C]*p*-chlorophenoxy-2-methylpropionic acid ([^{14}C]CFA) of specific activity 1290 MBq/mmol in 2.5 ml of 0.9% sodium chloride solution by intraperitoneal injection. Each rat received a further 5 ml of water by mouth immediately after the dose was given and was placed in a metabolic cage which allowed for the separate collection of urine and faeces. The urine container of each cage contained 15 ml of 0.5 *M* glycine buffer, pH 2.5, which was kept cold by contact with ice. Urine was collected for 16 h after the dose. The pooled urine was extracted twice with an equal volume of ice cold ethyl acetate and the combined ethyl acetate extracts were reduced to dryness at room temperature and reduced pressure in an evacuated centrifuge (Speed Vac Concentrator, Servant Instruments, NJ, U.S.A.). The residue was reconstituted in 3.5 ml of ice cold mobile phase (0.01 *M* citrate buffer, pH 2.5—acetonitrile, 65:35). Chromatographic purification of [^{14}C]-CFAG was carried out with 0.02-ml aliquots of the mobile phase containing [^{14}C]CFAG using the chromatographic system and conditions previously described [1] but with the above mobile phase.

Pooled fractions (2.5—3.0 min) containing the [^{14}C]CFAG were assayed for [^{14}C]CFA and [^{14}C]CFAG to determine their radiochemical purity and used as such for the preparation of calibration curves. Samples of [^{14}C]CFAG to be used in animal studies or for characterization by hydrolysis with β-glucuronidase were reduced to dryness over approximately 6 h by freeze-drying (Edwards High Vacuum Model EF3, Sussex, U.K.). [^{14}C]CFAG prepared by freeze-drying and that in mobile phase was stored at −20°C until used.

The freeze-dried material was dissolved in ice cold 0.9% sodium chloride solution (≈$5 \cdot 10^7$ dpm/ml) and two 0.05-ml aliquots were diluted with 0.45 ml of 0.1 *M* acetate buffer, pH 5.0, and incubated at 37°C for 2 h with approximately 500 Fishman units of β-glucuronidase (type H, Sigma, St. Louis, MO, U.S.A.). The pH of the solution was adjusted to 2.5 with 0.5 *M* buffer, pH 2.5, and assayed for [^{14}C]CFA and [^{14}C]CFAG.

Stability studies

Solutions of [^{14}C]CFAG were stored in separate glass tubes at −20°C which were then thawed and assayed for [^{14}C]CFAG and [^{14}C]CFA content over a period of six weeks. [^{14}C]CFAG solution (0.2 ml containing $4 \cdot 10^4$ dpm) was added to 2 ml of fresh rabbit plasma and incubated at 37°C. Plasma samples (0.2 ml) were taken at 1, 2.5, 5, 7.5, 10, 15, 20, 30, 45 and 60 min, after the addition and assayed for [^{14}C]CFAG in order to determine the stability of the glucuronide in fresh rabbit plasma.

Sample preparation

A schematic representation of the procedure is shown in Fig. 1. Each sample is split and an aliquot is counted in order to determine the total ^{14}C-labelled content in each sample which consists of [^{14}C]CFA and [^{14}C]CFAG. A second aliquot is extracted to determine the proportion in each sample of [^{14}C]CFA and [^{14}C]CFAG. The absolute radioactivity of CFA and CFAG is calculated as

$$\text{CFA (dpm/ml)} = T \times \frac{\text{CFA}}{\text{CFA} + \text{CFAG}} \quad (1)$$

and

$$\text{CFAG (dpm/ml)} = T \times \frac{\text{CFAG}}{\text{CFAG} + \text{CFA}} \quad (2)$$

where T is the total radioactivity in dpm/ml and CFA and CFAG are the disintegration rates of the chromatographic fractions corresponding to CFA and CFAG, respectively. Plasma or urine samples (0.2—0.5 ml) were placed in 4.5-ml capacity glass tubes containing 0.5 ml of ice cold 0.5 *M* glycine buffer, pH 2.5, and 1 ml cold ethyl acetate. All subsequent manipulations, with the exception of the ethyl acetate evaporation, were carried out either with the tubes in ice or at 4°C. The tubes were mixed by slow tumbling for 10 min and the ethyl acetate, separated by centrifugation at 1500 *g* for 3 min was transferred to a fresh tube and the process was repeated twice with fresh 1-ml portions of ethyl acetate. The combined ethyl acetate extracts were reduced to dryness over a period of approximately 20 min using the evacuated centrifuge, and the tube was rinsed with 0.1 ml of ice-cold mobile phase and kept on ice

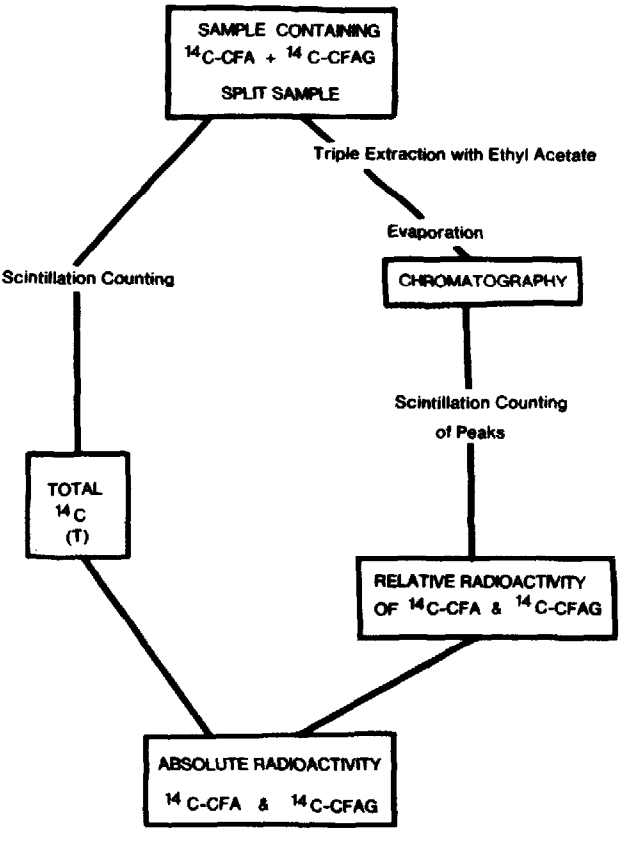

Fig. 1. A schematic representation of the sample preparation, chromatography and quantitation methods used in the essay.

until chromatographed. The entire content of each tube was injected into the high-performance liquid chromatograph (Waters, Model 6000A) via a Waters Model U6K injector (Waters Assoc., MA, U.S.A.). A 10-μm particle size reversed-phase column (Waters μBondapak C_{18}) was used with a mobile phase of 0.01 *M* citrate buffer, pH 2.5—acetonitrile (65:35). Eluent fractions corresponding to [^{14}C]CFAG and [^{14}C]CFA were collected into 25-ml capacity glass scintillation vials via a short length of stainless-steel tubing fitted to the column.

Scintillent, 15 ml (PCS, Amersham, IL, U.S.A.) was added to each tube and the count rates were determined in a scintillation counter (Model 6892, Searle Analytic, IL, U.S.A.). The count rates were converted to disintegration rates using the external standard method for quench correction. The disintegration rate of a 0.05—0.1-ml aliquot of biological fluid was also determined in a manner described above to obtain the total ^{14}C-labelled content of the sample.

Calibration

Calibration curves in plasma were prepared by adding known quantities of [^{14}C]CFAG (450—1000 dpm) and [^{14}C]CFA to plasma to produce ratios of [^{14}C]CFAG:[^{14}C]CFA in the range of 0.001—0.6. A similar procedure was used in urine but the range of ratios of [^{14}C]CFAG:[^{14}C]CFA was from 0.5—100. These samples were taken through the analysis and the observed ratios compared with those added to plasma or urine. Control samples which did not contain added ^{14}C were taken through the analytical procedure in order to determine background count rates which were subtracted from those of [^{14}C]CFA and [^{14}C]CFAG containing samples.

RESULTS AND DISCUSSION

Biosynthesis of [^{14}C]CFAG

[^{14}C]CFAG was prepared on two separate occasions. The percent of [^{14}C]CFA dose recovered in urine over 16 h was 92% and 71% on the first and second occasion, respectively. Direct injection of 0.01-ml aliquots under the chromatographic conditions described, revealed two fractions containing ^{14}C at 2.5—3 min and 6.5—7.5 min, the first corresponding to the retention time of authentic unlabelled CFAG under these conditions [1] and the second corresponding to the retention time of authentic [^{14}C]CFA.

In the first preparation, 77% of the urinary radioactivity was in the [^{14}C]CFAG fraction and 23% in the [^{14}C]CFA fraction, the corresponding values for the second preparation being 92% and 8%, respectively. When urine was directly injected, 1-min fractions collected for 30 min after the injection did not show any radioactive peaks greater than 1% of the total ^{14}C, other than those due to [^{14}C]CFA and [^{14}C]CFAG. After ethyl acetate extraction there was a mean of 5.2% of the urinary ^{14}C-activity remaining in the urine, which probably reflects the difficulty of complete removal of the ethyl acetate from a large volume of urine rather than the non-extraction of ^{14}C from the urine. The fractions from 2.5—3 min resulting from the injection of the ethyl acetate extract were collected and pooled. Subsequent chromatography of these pooled fractions showed that the first preparation had greater than 99% of the

^{14}C in the CFAG fraction, the remainder being CFA. The second preparation had 97.4% of the ^{14}C in the CFAG fraction. Aliquots of the fractions corresponding to [^{14}C]CFAG were reduced to dryness using the same conditions for each batch. Reanalysis of the first batch reconstituted in 0.9% sodium chloride solution showed that greater than 99% of the ^{14}C was associated with the CFAG fraction but in the second batch 85% of the ^{14}C was in the CFAG fraction, the remainder being in the CFA fraction. This reduction in radiochemical purity during solvent evaporation occurred in spite of apparently identical conditions being used on each occasion.

Analysis of the first batch indicated that all but 5% of the ^{14}C in the CFAG fraction before hydrolysis was associated with the CFA fraction after hydrolysis with β-glucuronidase and no other peaks were detected. Under the same conditions but in the absence of β-glucuronidase approximately 16% of ^{14}C in the CFAG fraction was associated with CFA representing non-enzymatic hydrolysis. In the second batch, 12.5% of the ^{14}C remained in the CFAG fractions after β-glucuronidase treatment. The activity associated with the [^{14}C]CFAG fraction but which was non-hydrolysable by β-glucuronidase may be due to rearrangement to non β-glucuronidase hydrolysable forms of the glucuronide in vivo or in vitro as previously reported for CFAG [2,3]. Taken together these data indicate that ^{14}C in the 2.5—3-min fraction is [^{14}C]CFAG, in that its retention time corresponds to authentic CFAG [1] and it is hydrolysed to a single product, CFA, by β-glucuronidase at a rate which exceeds non-enzymatic hydrolysis under the same conditions.

Stability studies

The ratio of [^{14}C]CFAG to CFA of the CFAG samples in 0.5 *M* glycine buffer, pH 2.5, at −20°C did not alter over a period of six weeks. Freeze-dried material stored at −20°C in anhydrous conditions also did not alter in the ratio of CFAG to CFA over this period.

In order to use the method in biological samples it was necessary to obtain an estimate of the hydrolysis rate of CFAG in biological fluids. Fig. 2 shows the loss of CFAG in fresh rabbit plasma at 37°C, the half-life for this process being 23 min. For this reason, in subsequent experiments with rabbits, blood samples were withdrawn and plasma was frozen in pH 2.5 buffer within 90 sec.

Analytical method

The approach outlined in Fig. 1 is valid only when all of the ^{14}C-labelled content in the biological fluid is due to CFA or CFAG. In preliminary experiments with plasma from rabbits administered [^{14}C]CFA, greater than 99% of the activity was removed by triple extraction with ethyl acetate and examination of this extract showed that this activity was associated only with CFA and CFAG. Similarly, when urine from rabbits administered [^{14}C]CFA was directly injected into the chromatographic system and 1-min fractions were collected for 30 min, radioactivity was found only in the 2.5—3 min and 6.5—7 min fractions, corresponding to CFAG and CFA respectively. It is unlikely that CFA would be converted to a lipophilic metabolite eluted substantially after CFA on a reversed-phase column. These findings are consistant with those of Caldwell et al. [4] who reported that rabbits administered [^{14}C]CFA excrete

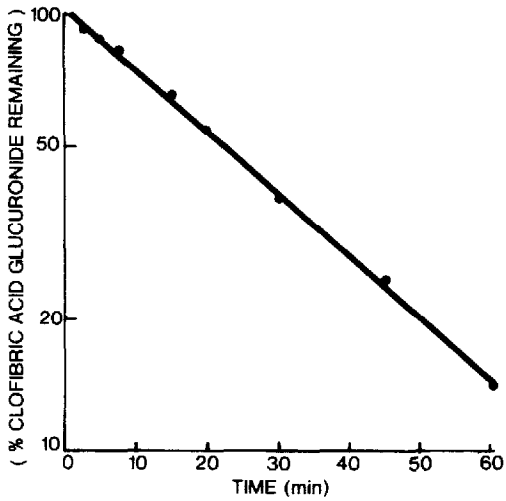

Fig. 2. The hydrolysis rate of CFAG (percent remaining, log. scale) in fresh rabbit plasma at 37°C. The half-life of this process is 23 min.

urinary ^{14}C, 9% as [^{14}C]CFA and 91% as CFAG. The approach outlined in Fig. 1 would not be valid in species such as dog or cat in which a substantial fraction of the dose is excreted as a taurine conjugate, unless the peak corresponding to this compound was separated and counted.

The sample preparation and chromatography present many opportunities for selective loss of CFA or CFAG and thus in order to validate the method it is necessary to demonstrate a correlation between the ratios of CFAG:CFA added to biological fluids and those estimated by the assay. Because of the extremely small ratios of CFAG to CFA anticipated in plasma (see Fig. 3) and the large ratios expected in urine, it was also important to ensure that there was efficient chromatographic separation of the two peaks with minimal carry over of radioactivity between them. The retention times of approximately 2.7 and 7 min for CFAG and CFA respectively meet these conditions, the column eluent between the 2.5—3 min fraction and the 6.5—7 min fraction falling to background values.

Because of the very wide range of values the regression equation for the relationship between the ratios of [^{14}C]CFAG to [^{14}C]CFA added to plasma and those estimated by the assay has been performed on the logarithms of the ratios in order to increase the weight of the lower values. If no weighting were used this would result in the low ratios making an insignificant contribution to the best fit regression equation. The regression equation in plasma is $y = 0.002 + 1.003x$ where y is the logarithm of the observed ratio and x is the logarithm of the added ratio, $r^2 = 0.9966$, $n = 35$ which indicates that the added and observed ratios in plasma are highly correlated with a slope of 1. The corresponding equation for urine is $y = -0.023 + 1.014x$, $r^2 = 0.991$, $n = 26$. These relationships indicate that in plasma over a range of CFAG:CFA ratios of the 0.001—0.6 and in urine over the range of 0.5—100 the method is able to accurately quantitate the concentrations of [^{14}C]CFAG and [^{14}C]CFA in biological fluids.

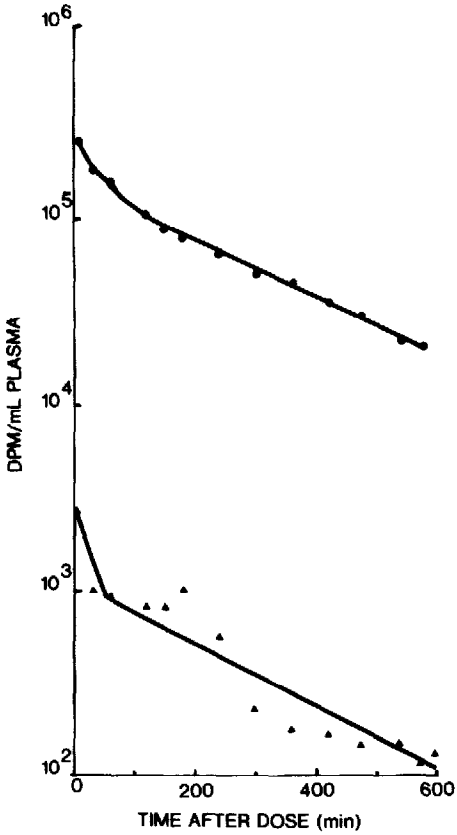

Fig. 3. The plasma concentration—time profiles of CFA (●—●) and CFAG (▲—▲) in a rabbit administered 1.85 MBq of [^{14}C]CFA.

The application of the method is illustrated in Fig. 3 which shows the plasma concentration—time profiles for CFAG and CFA resulting from the administration of 1.85 MBq of [^{14}C]CFA to a rabbit. The mean ratio in plasma of CFAG to CFA in this animal is 0.005.

The present method enables the quantitation of CFAG without prior hydrolysis to CFA and thus offers advantages over methods employing enzymatic or chemical hydrolysis in that it is more selective and sensitive [1]. The use of [^{14}C]CFA in conjunction with scintillation counting has increased the sensitivity of the method 100-fold relative to the previously described method using UV absorbance detection [1] and the only practical limitation to further increases in sensitivity is the specific activity of [^{14}C]CFA and its cost. The present method has allowed the quantitation of circulating plasma concentrations of [^{14}C]CFAG resulting from [^{14}C]CFA administration in an animal model which was not possible with the previous method based on UV absorbance detection. The application of the method to human studies would necessitate an evaluation of the ethical problems arising from the administration of radioisotopes to man.

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