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Subcellular distribution of metformin in rat liver

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Abstract—The subcellular localization of metformin was studied in livers of 18 h fasted rats treated orally with [^{14}C]metformin 20 μCi kg^{-1}, 50 mg kg^{-1}. Sequential determination of ^{14}C radioactivity showed that maximum concentrations of metformin in plasma (about 15 μmol L^{-1}) and liver (about 50 μmol kg^{-1}) were achieved at 30–60 min and approximately half-maximal concentrations were achieved at 4 h. At 30 min and 4 h after administration of [^{14}C]metformin, livers were removed and homogenized. Nuclear, mitochondrial and lysosomal, mixed membrane, and cytosolic fractions were separated by ultra-centrifugation. Distribution of ^{14}C was similar at both time points, being greatest in the cytosolic fraction (78% of total radioactivity, and 60–69% of relative specific activity). Small amounts of ^{14}C were associated with the other fractions. The total radioactivity and relative specific activity were respectively 2–3% and 7–8% in the nuclear fraction, 9–10% and 7–10% in the mitochondrial and lysosomal fraction, and 8–9% and 13–14% in the mixed membrane fraction. The higher concentration of metformin in liver compared with plasma suggests that metformin enters hepatocytes via a specific mechanism, and is distributed mainly within the cytosol.

Metformin (dimethylbiguanide) is an antihyperglycaemic agent used in the treatment of non-insulin-dependent diabetes mellitus. It lowers blood glucose concentrations by reducing the rate of intestinal glucose absorption, promoting glucose disposal and suppressing hepatic glucose production (Bailey & Nattrass 1988; Wilcock & Bailey 1991). There is evidence that metformin can potentiate insulin action, mainly at the postreceptor level (Bailey 1988), but the cellular mode of action of metformin remains to be established, and distribution of the drug within cells has not been studied. Since metformin is stable, not metabolized and excreted unchanged (Pentikainen et al 1979), [^{14}C]metformin was used in the present study to investigate the subcellular localization of the drug in rat hepatocytes after administration in-vivo.

Materials and methods

Animals. Adult male Wistar rats, ~120 g, were fasted for 18 h before experimentation.

In the first series of experiments [^{14}C]metformin hydrochloride (Lipha Pharmaceuticals, West Drayton, UK), specific activity 0·5 mCi mmol^{-1}, was administered orally (by intragastric gavage) as a bolus (20 μCi kg^{-1}, 50 mg kg^{-1}) dissolved in water (5 mL kg^{-1}). Anaesthesia was induced with sodium pentobarbitone (60 mg kg^{-1} i.p.) in different groups of rats 5 min before withdrawal of a blood sample (200 μL) from the hepatic portal vein and the inferior vena cava, and a liver biopsy (20 mg) at 0·5, 1, 2, 4, and 6 h. The biopsy sample was thinly sliced and thoroughly washed in saline. Plasma (100 μL) and tissue samples were added to 0·5 mL 1 M NaOH, heated to 90°C for 20 min and cooled. Ten mL of scintillant (Optiphase Hi-Safe II, LKB, Milton Keynes, UK) was added and vials were counted for ^{14}C-radioactivity using a Packard Tricarb scintillation counter.

In the second series of experiments [^{14}C]metformin hydrochloride was administered as above (20 μCi kg^{-1}, 50 mg kg^{-1}) and anaesthesia was induced before removal of the liver at 0·5 or 4 h. A polythene cannula (pp 20, Portex, Hythe, UK) was inserted into the hepatic portal vein and the hepatic vein was cut. Ice-cold heparinized saline (4 mL of sodium heparin 50 units mL^{-1} in 0·154 mol L^{-1} NaCl) followed by saline only (80 mL) were infused (16 mL min^{-1}) into the hepatic portal vein. The whole liver was removed, washed in ice-cold 0·25 mol L^{-1} sucrose, finely minced, homogenized, and subcellular fractions were prepared in 0·25 mol L^{-1} sucrose after the method of de Duve et al (1955). Four fractions were isolated: a nuclear fraction (N) sedimented at 900 *g* for 10 min; a mitochondrial and lysosomal fraction (ML) at 25000 *g* for 5 min; a mixed membrane fraction (MM) at 100000 *g* for 45 min; and a cytosolic fraction (C) being the supernatant of the MM fraction. Fractions were resuspended in 0·25 M sucrose. Samples of homogenate and each fraction were mixed with 1 M NaOH and treated as above for determination of ^{14}C-radioactivity. Samples of homogenate and fractions were analysed for protein (Lowry et al 1951), DNA (Anderson & Skegen 1977), acid phosphatase (test combination 125008, BCL, Lewes, UK), 5-nucleotidase (diagnostic kit 5 ND 6·5, number 265-A, Sigma Chemical Co, Poole, UK) and lactate dehydrogenase (LDH-P UV system kit 124885, BCL, Lewes, UK). Distribution of markers and [^{14}C]metformin were expressed as relative specific activity [enzyme activity or amount of DNA or ^{14}C per mg protein in the fraction expressed as a percentage of the sum of specific activities in all fractions (100%)].

Data were analysed by Student's *t*-test and differences were considered to be significant for $P < 0.05$.

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Results

Determination of ^{14}C-radioactivity in hepatic portal vein and inferior vena cava plasma and in liver at intervals after an oral dose of 50 mg kg^{-1} [^{14}C]metformin (Fig. 1) showed that maximum plasma concentrations of about 10^{-5} mol L^{-1} metformin were achieved after about 1 h. Concentrations were consistently higher in the hepatic portal vein than in the inferior vena cava ($P < 0.05$). Accumulation of metformin in the liver was maximal at about 30 min, and approximately half-maximum concentrations in both liver and plasma occurred at about 4 h. Liver accumulated 3–4 fold higher concentrations of [^{14}C]metformin than hepatic portal vein ($P < 0.05$) and inferior vena cava plasma ($P < 0.01$).

Data pertaining to fractionation of livers at 30 min and 4 h are shown in Table 1 and Fig. 2. The distributions of DNA, acid phosphatase, 5′-nucleotidase and LDH as markers of the N, ML, MM and C fractions, respectively, were essentially identical at the two times studied. The distribution of 5′-nucleotidase suggests a residue of membranes within the other fractions. However, rapid isolation of fractions was preferred to minimize any movement of metformin between fractions, and further purification was not attempted. In any case, the distribution of [^{14}C]metformin was not associated with the distribution pattern of 5′-nucleotidase. The distribution of [^{14}C]metformin closely parallelled the distribution of LDH, demonstrating that the greatest accumulation of [^{14}C]metformin was associated with the C (cytosolic) fraction (78% of total radioactivity, and 60–69% relative specific activity) at both time intervals studied.

The distribution of [^{14}C]metformin was not associated with the distribution of the other markers, and radioactivity in the N, ML and MM fractions was comparatively small (22% of total radioactivity).

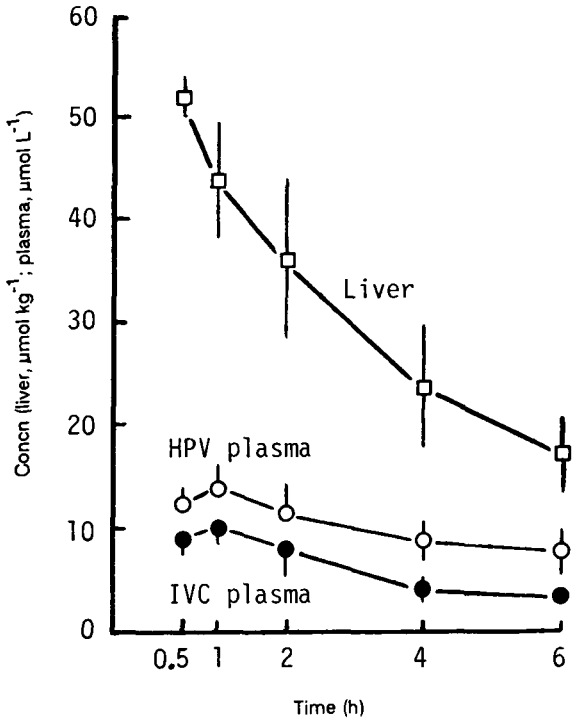

FIG. 1. Concentration of metformin in plasma of the hepatic portal vein (HPV) and inferior vena cava (IVC), and in liver of 18 h-fasted rats at intervals after oral administration of [^{14}C]metformin (20 μCi kg^{-1}, 50 mg kg^{-1}). Values are mean ± s.e.m. n = 4.

Table 1. [^{14}C]Metformin in plasma of the inferior vena cava and in liver of rats used for subcellular fractionation of liver. Rats were treated orally with metformin (20 μCi kg^{-1}, 50 mg kg^{-1}). Values are mean ± s.e.m. n = 4.

	30 min	4 h
Metformin concentration:		
plasma (μmol L^{-1})	14·0 ± 2·0	6·5 ± 1·8
liver (μmol kg^{-1})	49·6 ± 13·5	28·3 ± 7·3
Metformin concentration ratio (liver:plasma)	3·41 ± 0·66	4·42 ± 0·08
Recovery of administered dose in liver (%)	0·56 ± 0·12	0·26 ± 0·05

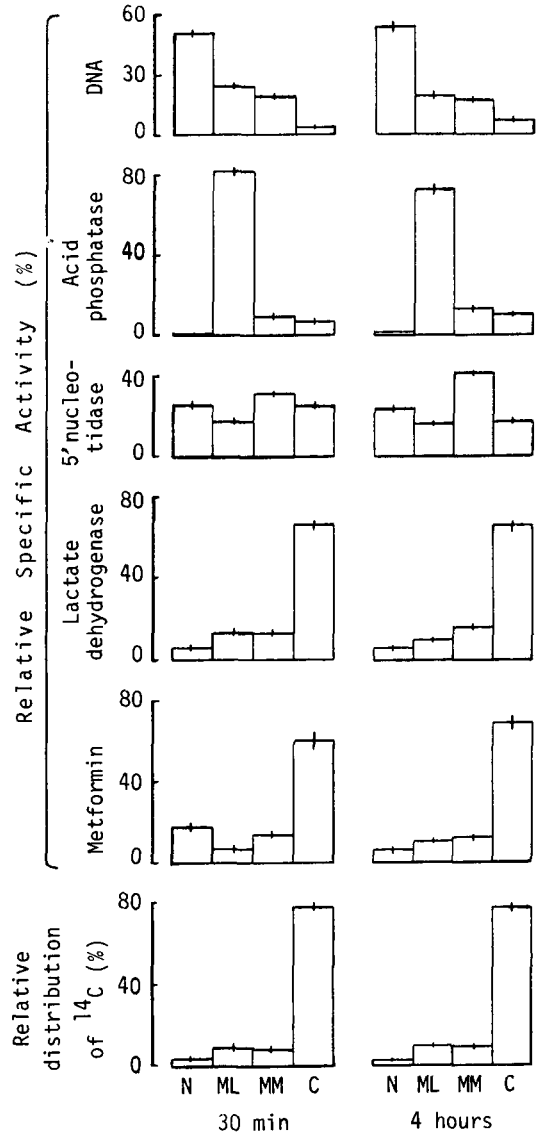

FIG. 2. Relative specific activity of markers and [^{14}C] metformin in nuclear (N), mitochondrial and lysosomal (ML), mixed membranes (MM) and cytosolic (C) fractions of liver, isolated at 30 min and 4 h after oral administration of [^{14}C]metformin (20 μCi kg^{-1}, 50 mg kg^{-1}) to 18 h fasted rats. Values are mean ± s.e.m. n = 4.

Discussion

Although metformin has been in clinical use for more than 30 years, its entry into cells of target tissues and its subcellular distribution have not previously been reported. There is clinical evidence that metformin is retained longer in whole blood than in plasma, indicating that higher concentrations of drug are associated with erythrocytes than with plasma (Tucker et al 1981). Recent unpublished studies in our laboratory have shown that metformin is accumulated by a range of target tissues including liver, and the present study has examined the association of metformin with subcellular components of liver in-vivo. The time taken for the fractionation procedures presents an interval for potential movement of metformin between fractions, and this must be borne in mind for the interpretation of the data. However, the same distribution of metformin at both of the time intervals studied suggests that metformin is associated predominantly with the cytosol of hepatocytes. This is consistent with the accumulation of a higher concentration of metformin in liver than in plasma (Fig. 1), and suggests a specific entry mechanism rather than diffusion. It has recently been noted that the secretion of metformin by the renal proximal tubule is competitively inhibited by the substituted guanidine, cimetidine (Somogyi et al 1987), indicating that metformin uses an active transmembranal transport mechanism in this tissue.

Alkyl-substituted biguanides bind to biological membranes in-vitro, and this property is conferred mainly by the hydrophobic side chains (Schafer 1979, 1980). In the metformin molecule these side chains are represented by two methyl groups, resulting in a greatly diminished affinity for the hydrophobic interior of membranes. This concurs with the present observation that less than 20% of the ^{14}C-radioactivity was associated with mitochondria, lysosomes and mixed membranes. These data corroborate the low binding affinity of metformin for mitochondrial membranes in-vitro (K_a = 0·012 mmol^{-1}; Schafer (1980)), which is one fiftieth of the binding affinity of phenformin

(phenethylbiguanide), and accords with physicochemical evidence that the partition coefficient of metformin in octanol/water is approximately one fortieth that of phenformin (Schafer 1980).

Metformin bears a single net positive charge at physiological pH, and it is likely that ionic interactions rather than hydrophobic effects contribute to its membrane binding properties. Ionic interactions may not be sufficiently strong to wholly withstand the present fractionation procedure, but the technique does provide clear evidence that metformin is associated predominantly with the cytosol of hepatocytes. We conclude that reports describing a membranal mode of action of long-chain substituted biguanides such as phenformin may not be directly extrapolated to metformin.

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Characterization of the variation between batches of Fast-Flo lactose using low frequency dielectric spectroscopy

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Abstract—The dielectric response of four batches of lactose has been measured over a frequency range of 10^4 to 10^{-2} Hz. The spectra corresponding to three of the batches were identical, while the fourth showed a marked reduction in response. This particular batch has also been reported to exhibit longer disintegration times than the other three when formulated as a tablet. The potential use of dielectric spectroscopy as a means of screening batches of pharmaceutical materials is discussed.

In a recent study on inter-batch variation between Fast-Flo lactose samples (Boyd et al 1989), we demonstrated that tablets produced from a particular batch of lactose yielded considerably slower disintegration times than did other, supposedly identical samples. However, no significant physical differences could be found between the materials using optical microscopy or X-ray powder diffraction. Measurements of adsorbed water, water of crystallization, tap density and angle of repose yielded similar results for all four samples. The study therefore highlighted some of the problems associated with inter-batch variation, in particular the difficulty in finding a technique which can identify potential problems at an early stage.

In the present investigation, the same four batches of lactose were examined using low frequency dielectric spectroscopy (DISP) in order to ascertain whether any differences could be detected. DISP has been employed in the analysis of several pharmaceutical systems, some of which have been reviewed by Craig et al (1990). The method involves the application of a small alternating voltage to a sample and the subsequent measurement of the energy stored (as capacitance) and lost (as dielectric loss) over a range of frequencies. The resulting spectra may yield information on the structure and behaviour of a wide variety of systems. For a more detailed description of the theoretical aspects of the technique, the reader is referred to Dissado & Hill (1979) and Hill & Jonscher (1983).

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Materials and methods

Samples taken from four batches of Fast-Flo lactose (Foremost Whey Products, Wisconsin, USA), designated Batches A, B, C and D, were analysed as received. Three hundred milligrams of each material was compressed in an IR press to form discs of diameter 13 mm and thickness $1{\cdot}58 \pm 0{\cdot}03$ mm. Each sample was placed between two platinum electrodes (area 81 mm^2) in a Perspex dielectric cell, held at 298K in a cryostat (Oxford Instruments Ltd, Oxford). An AC signal of 0·5 V was generated by a frequency response analyser (FRA, Solartron, Hampshire) and passed through the sample via a Chelsea Interface (Chelsea Dielectrics Group, London). The returning signal was analysed by the FRA and the results presented graphically in terms of the capacitance and dielectric loss over a frequency range of 10^4 to 10^{-2} Hz. At least three measurements were made at each frequency and the average calculated automatically. Spectra