In summary, this investigation suggests that the oxazolidine derivative of propranolol is a prodrug which is hydrolysed stereoselectively to propranolol by hepatic post-mitochondrial supernatant. The (*S*)-form of the prodrug is more stable in the biological system than its (*R*)-form.

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REFERENCES

1. Jamali F, Mehvar R and Pasutto FM, Enantioselective aspects of drug action and disposition: therapeutic pitfalls. *J Pharm Sci* **78**: 695–715, 1989.
2. Walle T, Webb J, Bagwell E, Walle K, Daniell H and Gaffney T, Stereoselective delivery and actions of beta receptor antagonists. *Biochem Pharmacol* **37**: 115–125, 1988.
3. Howe R and Shanks RG, Optical isomers of propranolol. *Nature* **210**: 1336–1338, 1966.
4. Barrett AM and Cullum VA, The biological properties of the optical isomers of propranolol and their effects on cardiac arrhythmias. *Br J Pharmacol* **34**: 43–55, 1968.
5. Rahm K, Hawlina A, Kersting F and Planz G, Studies on the antihypertensive action of the optical isomer of propranolol in man. *Naunyn Schmiedebergs Arch Pharmacol* **286**: 319–323, 1974.
6. Linder W, Rath M, Stoschitzky K and Semmelrock HJ, Pharmacokinetic data of propranolol enantiomers in a comparative human study with (*S*); and (*R,S*)-propranolol. *Chirality* **1**: 10–13, 1989.
7. Walle UK, Thibodeaux H, Privitera PJ and Walle T, Stereochemistry of tissue distribution of racemic propranolol in the dog. *Chirality* **1**: 192–196, 1989.
8. Lo M-W, Effeney DJ, Pond SM, Silber BB and Riegelman S, Lack of gastrointestinal metabolism of propranolol in dogs after portacaval transition. *J Pharmacol Exp Ther* **222**: 512–515, 1982.
9. Iwamoto K and Watanabe J, Dose-dependent presystemic elimination of propranolol due to hepatic first-pass metabolism in rats. *J Pharm Pharmacol* **37**: 826–828, 1985.
10. Garceau Y, Davis I and Hasegawa J, Plasma propranolol levels in beagle dogs after administration of propranolol hemisuccinate ester. *J Pharm Sci* **67**: 1360–1363, 1978.
11. Anderson BD, Chu WW and Galinsky RE, Reduction of first-pass metabolism of propranolol after oral administration of ester prodrugs. *Int J Pharm* **43**: 261–265, 1988.
12. Takahashi K, Haginaka J, Tamagawa S, Nishihata T, Yasuda H and Katagi T, Stereoselective hydrolysis of O-acetyl propranolol as prodrug in human serum. *J Pharm Pharmacol* **42**; 356–357, 1990.
13. Kamal A, Metabolism of ethyl 2-carbamoyloxybenzoate (4003/2), a prodrug of salicylic acid, carsalam and salicylamide. *Biochem Pharmacol* **40**: 1669–1671, 1990.
14. Crowther AF and Smith LH, β-Adrenergic blocking agents: II. Propranolol and related 3-amino-1-naphthoxy-2-propranols. *J Med Chem* **11**: 1009–1013, 1968.
15. Hermansson J, Resolution of racemic aminoalcohols (β-blockers), amines, acids as enantiomeric derivatives using chiral α_1-acid glycoprotein column. *J Chromatogr* **325**: 379–384, 1985.
16. Hyne JB, Nuclear magnetic resonance spectra and configuration. The NMR spectra of diastereoisomeric heterocyclic derivatives of the ephedrines. *J Am Chem Soc* **81**: 6058–6061, 1959.
17. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.

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The fate of intravenously administered biotin-labelled hyaluronidase in the rat

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Traditionally, mammalian testicular hyaluronidases (hyaluronate 4-glycanohydrolase, EC 3.2.1.35) have been indicated in the treatment of a variety of clinical conditions [1] and are often used to enhance the intramuscular or subcutaneous administration of a variety of drugs. More recently, the efficacy of the enzyme has been evaluated for the treatment of myocardial infarction [2] and other conditions [3]. Efficacy remains to be established although there have been clinical improvements in some of the patients with myocardial infarction and a reduction in cumulative mortality [4–6]; however results from other studies are less clear [7, 8].

A major problem with the intravenous administration of hyaluronidase is the rapid removal of the enzyme from the bloodstream; the serum half-life in humans is approximately 3.2 min [9]. Similar values for half-life have been measured in rats using ^{125}I-labelled hyaluronidase where the bulk of the enzyme has accumulated in the liver within 10 min of administration [10]. Reducing the accumulation of enzyme by liver with a resultant increase in serum half-life should improve efficacy. The presence of three high-mannose type oligosaccharide subunits per polypeptide chain of hyaluronidase [11] is probably the reason for rapid uptake by liver. Previous studies with ^{125}I-labelled enzyme have shown that pre-administration of mannans significantly increased the serum half-life [10].

Preliminary studies have indicated that it is possible to label hyaluronidase with biotin as an alternative to ^{125}iodine [12]. This would allow the distribution and uptake of the enzyme to be monitored using an independent method based on detection with streptavidin-conjugates. In this paper we report on the use of biotin-labelled hyaluronidase

for the analysis of the serum kinetics, tissue distribution and sub-cellular localization of normal and deglycosylated enzyme in the rat.

Materials and Methods

A highly purified (>40,000 I.U./mg) preparation of hyaluronidase (trade names of GL enzyme and Hyalosidase) as used in clinical trials in the U.K. [4–6] was donated by Biorex Laboratories, London, U.K. All other chemicals were obtained from sources identified previously [13].

Hyaluronidase was biotinylated using a 2000-fold molar excess of biotin-*N*-hydroxysuccinimide ester to enzyme, resulting in preparations that contained 35 ± 5 mol biotin/mol enzyme (mean ± SD of three experiments) [13]. Samples of native and biotinylated enzyme were deglycosylated using endoglycosidase F [13].

Hyaluronidase activity was measured by quantitation of the release of *N*-acetyl hexosamine reducing equivalents from a solution of potassium hyaluronate [14]. Acid phosphatase [15], aryl sulphatase C [16], cytochrome oxidase [17] and lactate dehydrogenase [18] were assayed using published methods.

Protein was quantitated by the Folin method using bovine serum albumin as a standard [19]. DNA was assayed with diphenylamine using calf thymus DNA as a reference [20].

A sandwich assay technique was used for the estimation of biotinylated hyaluronidase. The wells (four to eight wells/sample) of a microtitre plate were primed by overnight incubation at 4° with 50 μL/well of streptavidin (5 μL/mL of phosphate buffered saline, pH 7.2, PBS) and washed three times with PBS. Unoccupied protein-binding sites were blocked by incubation (30 min at 25°) with 100 μL/well of bovine serum albumin (10 mg/mL) and the wells washed with PBS. Samples (50 μL) of biotinylated hyaluronidase were added to the wells and left for 1 hr at 25°. The wells were rinsed with PBS prior to the addition of 50 μL/well of peroxidase-conjugated streptavidin (1 : 1000 dilution in PBS). After 1 hr at 25° the wells were rinsed and the binding of the conjugate determined using 1,2-benzenediamine as the substrate for the peroxidase reaction [13].

The serum half-life and tissue distribution of both native and biotinylated hyaluronidase in rats was determined as described previously [10]. Preliminary experiments established that optimum and reproducible release of biotinylated enzyme from tissue was achieved by sonication for 1 min/g of tissue. Subcellular fractions of liver were prepared [21] and analysed for DNA, protein, marker enzymes and biotinylated hyaluronidase. Samples of subcellular fractions were prepared for electron microscopy by preliminary fixation in 1.5% (v/v) glutaraldehyde, followed by fixation, embedding and sectioning [22].

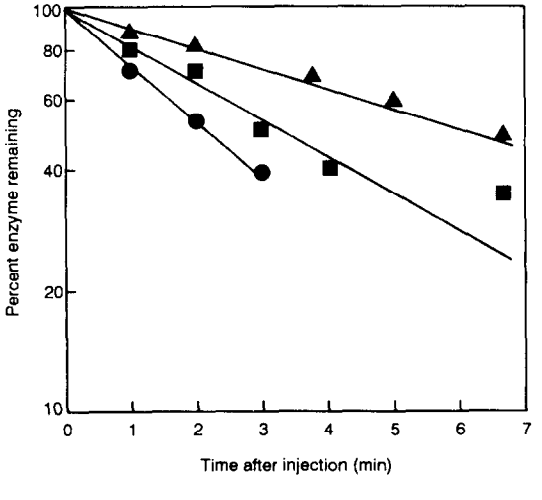

Fig. 1. Estimation of the half-life of biotinylated hyaluronidase in rat blood. Half-lives were estimated for the enzyme alone (●), enzyme + mannans (■) and deglycosylated hyaluronidase (▲).

Results and Discussion

The solid-phase sandwich assay proved to be useful for the determination of biotinylated hyaluronidase and was less prone to interference by exogenous proteins than other methods. In the presence of tissue extracts the sensitivity of the assay allowed the detection of between 25 and 500 ng of biotinylated hyaluronidase/mL. In all cases control assays were performed to compensate for basal levels of biotin present in tissues.

The serum half-lives of both native and biotinylated hyaluronidase were 2.5 ± 0.2 and 2.4 ± 0.4 min, respectively (Fig. 1), which is consistent with previous results [10]. A significant increase in half-life was observed if mannan was pre-administered (native enzyme, $t_{\frac{1}{2}} = 3.4 \pm 0.4$ min; biotinylated enzyme, $t_{\frac{1}{2}} = 3.9 \pm 0.3$ min) or the hyaluronidase was deglycosylated (native enzyme, $t_{\frac{1}{2}} = 7.0 \pm 2.1$ min; biotinylated enzyme, $t_{\frac{1}{2}} = 7.0 \pm 0.9$ min). Therefore, the conjugated biotin and the concomitant partial loss of enzyme activity does not appear to affect either the rate or the mode of uptake of hyaluronidase. Furthermore, the estimated half-lives are comparable with those previously obtained using ^{125}I-labelled hyaluronidase [10].

The tissue distribution of biotin remaining in the rat following i.v. injection of biotinylated hyaluronidase was measured 10 min after the administration of the enzyme. This time was selected to allow direct comparison with previous results using ^{125}I-labelled hyaluronidase [10]. The bulk of the administered biotin (51.3 ± 4.4%) accumulated in the liver with measurable quantities in the kidneys (3.3 ± 0.3%) and spleen (0.9 ± 0.1%). Significant levels of endogenous biotin prevented quantitation in other tissues of interest. The accumulation of hyaluronidase in the liver was significantly reduced by either pre-administration of mannan (15.7 ± 4.4%) or the use of deglycosylated enzyme (10.2 ± 5.3%). This indicates that the oligosaccharide units present on hyaluronidase are the major determinant in the uptake of enzyme from the circulation.

Sub-cellular fractionation established that biotinylated hyaluronidase was primarily associated with the lysosomal and mitochondrial fractions (Fig. 2). The most likely explanation is that biotinylated hyaluronidase is endocytosed by the liver and accumulated in the lysosomes. The apparent association of biotinylated hyaluronidase with the mitochondria probably resulted from incomplete resolution of the mitochondrial and lysosomal fractions as judged by the observed distribution of acid phosphatase, a lysosomal marker enzyme. This may be due to an increase in density of the lysosomes following endocytosis of biotinylated hyaluronidase. A similar phenomenon has been reported following endocytosis of invertase and mannans by rat liver [23, 24].

Electron microscopy was used to investigate whether abnormally dense lysosomes had sedimented with the mitochondrial fraction. In the lysosomal pellicle lysosomes were approximately 0.14 μm in diameter and some were in the process of fusion. The mitochondrial pellicle contained some lysosomes and these were approximately 0.45 μm in diameter. In addition, some preliminary experiments using polyclonal anti-hyaluronidase antibodies and gold-conjugated second antibody have indicated that hyaluronidase is accumulated only by lysosomes [25]. No attempt has been made to establish whether a particular cell type was

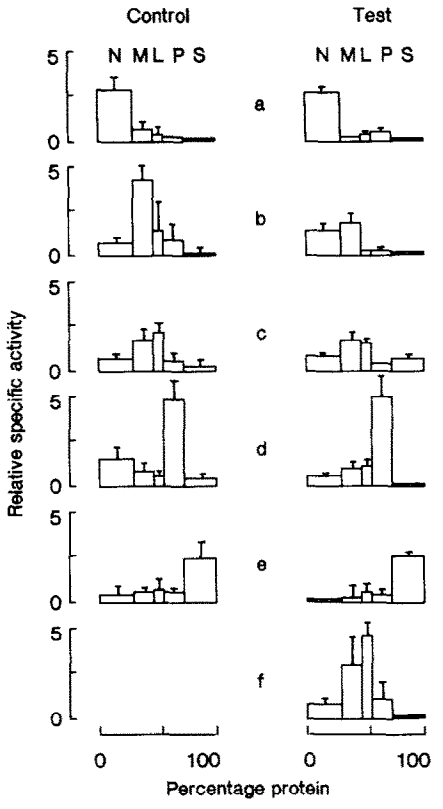

Fig. 2. The intracellular distribution of biotinylated hyaluronidase in rat liver. N, M, L, P, S represent the nuclear, mitochondrial, lysosomal, microsomal and cytoplasmic fractions, respectively. Histograms show the distribution of (a) DNA, (b) cytochrome oxidase, (c) acid phosphatase, (d) aryl sulphatase C, (e) lactate dehydrogenase and (f) biotinylated hyaluronidase. The results represent the mean ± SD of three experiments.

responsible for the endocytosis of hyaluronidase. However, as the Kupfer cells contain the majority of mannose-specific endocytic receptors in the liver [26, 27] it might be predicted that these cells bind and internalize most of the enzyme.

The labelling of hyaluronidase with biotin has proved to be useful for monitoring the fate of the intravenously administered enzyme. Although the detection of enzyme is less sensitive using biotin instead of ^{125}I-labelling, comparison of the two methods allows any effects of the labels to be eliminated. Any influence of the labels on the fate of hyaluronidase appears to be negligible as both the biotinylated and iodinated preparations of enzyme showed similar serum kinetics and tissue distribution.

Deglycosylation of the hyaluronidase could prove to be an effective way of increasing the serum half-life of the enzyme. The half-life of the deglycosylated enzyme was approximately three times greater than the native enzyme. A further increase in half-life may be possible as endoglycosidase F removes only 66% of the carbohydrate associated with hyaluronidase [13]. The use of a combination of endoglycosidases of appropriate specificity may allow the complete removal of both the major and minor oligosaccharide subunits [28]. The therapeutic effects of the administration of deglycosylation preparations now require further evaluation.

In summary, a highly purified commercial preparation of bovine testicular hyaluronidase (GL enzyme, Hyalosidase) was labelled with biotin. This preparation was administered intravenously into rats and the serum half-life determined by measurement of enzyme activity and detection of biotin. Tissue distribution studies established that the major site of uptake was the liver (51.3%). The rapid uptake of hyaluronidase by the liver was reduced by pre-administration of mannan or by deglycosylation of the enzyme. Subcellular fractionation of the liver revealed that most of the administered hyaluronidase was associated with the lysosomes. These results indicate that hyaluronidase uptake by the liver is mediated by mannose-specific receptors and that the enzyme is internalized into the lysosomes.

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REFERENCES

1. Evans BK, Gacesa P and Olavesen AH, Hyaluronidase. In: *Therapeutic Drugs—A Clinical Pharmacopoeia* (Ed. Dollery CT), Churchill Livingstone, Edinburgh, in press.
2. Maroko PR, Hillis LD, Muller JE, Tavazzi L, Heyndrickx GR, Ray M, Chiariello M, Distante A, Askenaski J, Salerno J, Carpentier J, Reshetnaya NI, Radvany P, Libby P, Raabe DS, Chazov EI, Bobba P and Braunwald E, Favourable effects of hyaluronidase on electrocardiographic evidence of necrosis in patients with acute myocardial infarction. *N Engl J Med* **296**: 898–903, 1977.
3. Elder JB, Raftery AT and Cope V, Intra-arterial hyaluronidase in severe peripheral arterial disease. *Lancet* **i**: 648–649, 1980.
4. Flint DJ, DeGiovani J, Cadigan PJ, Lamb P and Pentecost BL, Effect of GL enzyme (a highly purified form of hyaluronidase) on mortality after myocardial infarction. *Lancet* **i**: 871–874, 1982.
5. Henderson A, Campbell RWF and Julian DG, Effect of a highly purified hyaluronidase preparation (GL enzyme) on electrocardiographic changes in acute myocardial infarction. *Lancet* **i**: 874–876, 1982.
6. Saltissi S, Robinson PS, Coltart DJ, Webb-Peploe MM and Croft DN, Effects of early administration of a highly purified hyaluronidase preparation (GL enzyme) on myocardial infarct size. *Lancet* **i**: 867–871, 1982.
7. MILIS Study Group, Hyaluronidase therapy for acute myocardial infarction: Results of a randomized, blinded multicenter trial. *Am J Cardiol* **57**: 1236–1243, 1986.
8. Julian DG, Pentecost BL, Simpson JM, Smith RH, Cadigan PJ and Petri MP, Intravenous (IV) hyaluronidase in suspected acute myocardial infarction (MI). *Circulation* **72**: (Suppl III): 222, 1985.
9. Wolf RA, Chaung LY, O'Hara D, Smith TW and Muller JE, The serum kinetics of bovine testicular hyaluronidase in dogs, rats and humans. *J Pharmacol Exp Ther* **222**: 331–337, 1982.
10. Earnshaw JS, Curtis CG, Powell GM, Dodgson KS, Olavesen AH and Gacesa P, The fate of intravenously

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administered highly purified bovine tesitcular hyaluronidase (Hyalosidase) in the rat. *Biochem Pharmacol* **34**: 2199–2203, 1985.
11. Michalski J-C, Olavesen AH, Winterburn PJ, Pope DJ and Strecker G, The structure of the major oligosaccharide unit of bovine testicular hyaluronidase. *Biochem Soc Trans* **12**: 655–656, 1984.
12. Lace D, Dodgson KS, Gacesa P and Olavesen AH, The preparation of biotin-labelled hyaluronidase. *Biochem Soc Trans* **15**: 402–403, 1987.
13. Lace D, Olavesen AH and Gacesa P, The effects of deglycosylation on the properties of native and biotinylated bovine testicular hyaluronidase. *Carbohydr Res* **208**: 306–311, 1990.
14. Gacesa P, Savitsky MJ, Dodgson KS and Olavesen AH, A recommended procedure for the estimation of bovine testicular hyaluronidase in the presence of human serum. *Anal Biochem* **118**: 76–84, 1981.
15. Gianetto R and de Duve C, Tissue fractionation studies. 4. Comparative study of the binding of acid phosphatase, glucuronidase and cathepsin by rat-liver particles. *Biochem J* **59**: 433–445, 1955.
16. Milsom DW, Rose FA and Dodgson KS, The specific assay of arylsulphatase C, a rat liver microsomal marker enzyme. *Biochem J* **128**: 331–336, 1972.
17. Cooperstein SJ and Lazarow A, A microspectrophotometric method for the determination of cytochrome oxidase. *J Biol Chem* **189**: 665–670, 1951.
18. Delbruck A, Schimassek H, Bartsch K and Bucher Th, Enzym-verteilungsmuster in einigen organen und in experimentellen tumoren der ratte und der maus. *Biochem Z* **331**: 297–311, 1959.
19. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
20. Leyva A Jr and Kelley WN, Measurement of DNA in cultured human cells. *Anal Biochem* **62**: 172–179, 1974.
21. de Duve C, Pressman BC, Gianetto R, Wattiaux R and Applemans F, Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem J* **60**: 604–617, 1955.
22. Baudhuin P, Evrard P and Berthet J, Electron microscopic examination of subcellular fractions. I. The preparation of representative samples from suspensions of particles. *J Cell Biol* **32**: 181–191, 1967.
23. Jadot M, Wattiaux-De Coninck S and Wattiaux R, Effect on lysosomes of invertase endocytosed by rat-liver. *Eur J Biochem* **151**: 485–488, 1985.
24. Wattiaux R, Wattiaux-De Coninck S, Dubois F and Braun A, Influence of the injection of mannan on rat liver lysosomes. *Biochem Soc Trans* **15**: 435, 1987.
25. Lace D, Gacesa P, Hann AC and Olavesen AH, Lysosomal accumulation of intravenously administered hyaluronidase. *Biochem Soc Trans* **18**: 490–491, 1990.
26. Schlesinger PH, Doebber TW, Mandell BF, White R, De Schryver K, Rodman JS, Miller MJ and Stahl P, Plasma clearance of glycoproteins with terminal mannose and *N*-acetylglucosamine by liver non-parenchymal cells. Studies with β-glucuronidase, *N*-acetyl-β-D-glucosaminidase, ribonuclease B and agalacto-orosomucoid. *Biochem J* **176**: 103–109, 1978.
27. Hubbard AL, Wilson G, Ashwell G and Stukenbrok H, An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. I. Distribution of ^{125}I-ligands among the liver cell types. *J Cell Biol* **83**: 47–64, 1979.
28. Hsieh P, Rosner MR and Robbins PW, Host-dependent variation of asparagine-linked oligosaccharides at individual glycosylation sites of Sindbis virus glycoproteins. *J Biol Chem* **258**: 2548–2561, 1983.