International Journal of Pharmaceutics, 61 (1990) 219–223
Elsevier

IJP 02065

Effect of microencapsulated acetylsalicylic acid on glycosylation of human serum proteins in vitro

Dubravka Juretić, Ivana Čepelak, Vesna Jalšenjak, Tihana Žanić-Grubišić, K. Lipovac and I. Jalšenjak

Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia (Yugoslavia)

(Received 23 November 1989)
(Accepted 12 December 1989)

Key words: Acetylsalicylic acid microcapsule; Protein glycosylation

Summary

Acetylsalicylic acid microencapsulated in a biocompatible polymer (poly(lactic acid)) was used for the inhibition of human serum glycosylation in comparison with the free drug. At two levels of glucose and acetylsalicylic acid, the inhibition of glycosylation was markedly enhanced for the microencapsulated drug. The observed increase in inhibition was ascribed either to delayed hydrolysis of the drug or removal of glucose to microcapsules.

Introduction

Among the various pharmacological effects of acetylsalicylic acid, its hypoglycaemic effect in patients with diabetes mellitus has been realized (Reid et al., 1957) but remains incompletely understood. Several authors have studied the inhibition of protein glycosylation by acetylsalicylic acid. Glycosylation is an irreversible non-enzymatic process in which a body protein with its free amino groups reacts with carbonyl groups of blood glucose to yield, after rearrangement, a stable ketoamine product. As the rate of glycosylation depends on the plasma concentration of glucose and the levels of glycosylated proteins are higher in patients with diabetes, Kennedy and Lyons (1989) have suggested that the formation of advanced end products of glycosylation may provide a link between hyperglycaemia and chronic diabetes complications. On the other hand Hawkins et al. (1969) have shown that acetylsalicylic acid acetylates human serum proteins. Rendall et al. (1986) have confirmed that the drug, but not salicylic acid, effectively inhibits glycosylation of albumin by prior acetylation of protein amino groups.

Initially, large doses of acetylsalicylic acid (> 5 g/day) were shown to have a hypoglycaemic effect (Reid et al., 1957), but more recently, smaller drug doses (about 1 g/day) have come into use, since they are less likely to produce side effects and complications (Alberti and Press, 1982; Hopper et al., 1985). Taking into account the relatively high doses of acetylsalicylic acid and the duration of possible administration, as well as the risk of drug-induced damage to the gastric mucosa and/or gastrointestinal bleeding in humans (Jick,

Correspondence: D. Juretić, Faculty of Pharmacy and Biochemistry, University of Zagreb, 41000 Zagreb, A Kovačića 1, Croatia, Yugoslavia.

1981), it appeared appropriate to assess the effect of microencapsulated acetylsalicylic acid on glycosylation of serum proteins in vitro. The use of microcapsules to achieve various objectives (such as environmental protection, sustained or controlled release, stability enhancement) is well established, and acetylsalicylic acid was one of the first candidates for microencapsulation (Deasy, 1984).

The main objective of the present investigation was to evaluate the effect of microencapsulated acetylsalicylic acid on glycosylation of serum proteins in vitro in comparison with the free (unencapsulated) drug. The microcapsules were prepared from a biocompatible polymer poly(lactic acid) by the so-called solvent evaporation method. In addition, acetylsalicylic acid release from microcapsules was followed by two methods in vitro.

Materials and Methods

Materials

All chemicals were of analytical grade. Poly(lactic acid) (mol. wt. 33000, m.p. 150 °C) was supplied by Polysciences (Warrington, PA). Fructosamine test (Roche) was a sample provided by the manufacturer.

Microcapsule preparation and characterization

Microencapsulation was carried out by a modification of the solvent evaporation method as follows. Poly(lactic acid) (1500 mg) was dissolved in dichloromethane (30 ml) and acetylsalicylic acid (300 mg) was added to the solution. The mixture was rapidly transferred to 0.3% methylcellulose aqueous solution (300 ml) and vigorously stirred until all the dichloromethane had evaporated and microcapsules were formed. The product was decanted off and washed several times with water, filtered off and the product air dried.

An optical microscope was used to determine the capsule size obtained, the observed size range being from 20 to 230 μm. The drug content of microcapsules was 16.6% according to Method I described in the next section.

Release of acetylsalicylic acid from microcapsules

Method I The drug release into water from poly(lactic acid) microcapsules was followed by applying the flask and paddle method. Microcapsules (500 mg) were placed in a round-bottomed flask containing 1000 ml water prewarmed to 37 °C (stirring speed 50 rpm). Samples (2 ml each) were removed at intervals and the released drug was determined spectrophotometrically (274 nm) after appropriate dilution with concentrated ethanol had been performed. The drug content of capsules was calculated on the basis of this determination.

Method II To 30 ml of non-diabetic human serum in a cuvette 135 mg of glucose and the amount of microcapsules containing 18 mg acetylsalicylic acid were added. Sodium azide (3 mg) was added as a preserving agent. The mixture was incubated at 37 °C. At intervals 0.1-ml samples were withdrawn, and the concentration of salicylates was determined by a modification of Trinder's method according to Richterich and Colombo (1984).

Estimation of protein glycosylation

Assessment of the total glycosylated serum proteins was performed by the fructosamine assay. The test is based on the reduction of nitroblue tetrazolium chloride to coloured diformazan by the ketoamine formed during glycosylation (Johnson et al., 1982). Results are expressed as equivalents to the chosen calibration standard 1-deoxy-1-morpholinofructose (DMF).

Two pools of non-diabetic human serum mixtures were prepared as follows: mixtures A (1, 30 ml serum + 135 mg glucose; 2, 30 ml serum + 135 mg glucose + 18 mg acetylsalicylic acid; 3, 30 ml serum + 135 mg glucose + microcapsules containing 18 mg acetylsalicylic acid) and mixtures B with double the amount of glucose (270 mg) and acetylsalicylic acid (36 mg). For the sake of comparison, a mixture of serum, glucose and poly(lactic acid) was investigated. Sodium azide (3 mg) was added as a preserving agent. 0.1 ml of a serum mixture was removed and the total glycosylated serum proteins were estimated using a commercial fructosamine test (Roche) by a standard procedure.

Results and Discussion

The microcapsules obtained were of roughly spherical shape, capsules ranging in diameter from about 20 to 230 μm. They were used without size fractionation, and had a drug content of 16.6%. The acetylsalicylic acid release from microcapsules (or free drug dissolution) determined via two different methods was compared and some interesting features were observed. Method I which is a modification of a usual 'flask and paddle method' often employed in microcapsule research, showed the process of dissolution of free acetylsalicylic acid to be complete within a few minutes of commencing an experiment (Fig. 1), while the drug release from microcapsules was prolonged for several hours. Rather low $t_{1/2}$ values in vitro and the subsequent sustained release can be explained by the spatial distribution of drug within a microcapsule matrix. A substantial amount of drug is situated in the proximity of the microcapsule surface, producing an early burst effect (Deasy, 1984), the remaining fraction of drug being released afterwards in a true gradual manner.

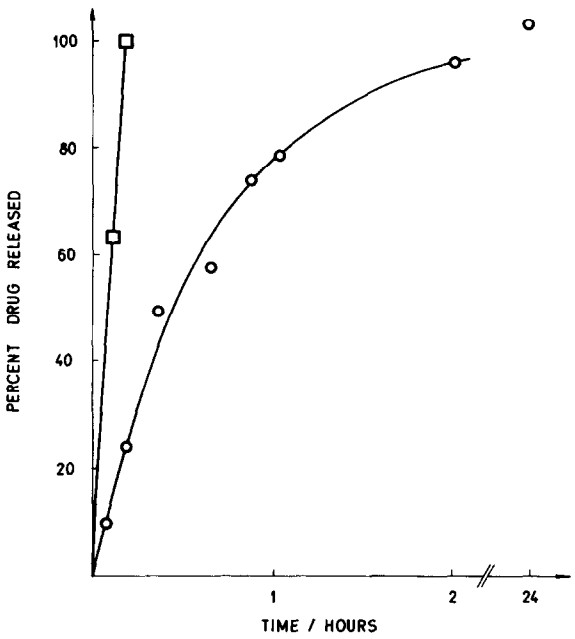

Fig. 1. In vitro release of acetylsalicylic acid (Method I). ○, poly(lactic acid) microcapsules; □, free drug.

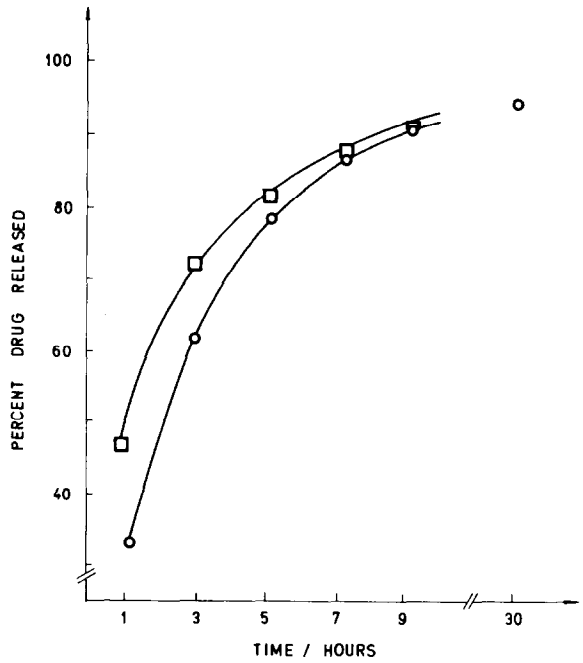

Fig. 2. In vitro release of acetylsalicylic acid (Method II). ○, poly(lactic acid) microcapsules; □, free drug.

Method II was chosen in order to resemble the experimental conditions for protein glycosylation. The results from that method indicate that the non-sink conditions and/or lack of stirring during drug release influence the pattern of drug release. The $t_{1/2}$ values for free and microencapsulated drug were 1 and 2 h, respectively (Fig. 2). Differences recorded by both methods conform to the well-known situation that the particular methodology influences the release pattern from microcapsules (Jalšenjak, 1990). Fig. 3 shows the release pattern (obtained using Method II) for the entire period of 7 days. The amount of acetylsalicylic acid, or more precisely, salicylates (due to hydrolysis) of the drug once it is released from microcapsules and their protection, reaches about 80% during the first day, thereafter increasing slowly up to about 90%. In none of the cases did the total amount of drug throughout the 7 days attain the initial value of the amount encapsulated.

Although a definite evaluation of the fructosamine assay is still lacking (Koskinen et al. 1987; Winocour et al. 1987; Jerntorp et al. 1988; Phillipou et al. 1988), we have selected this method

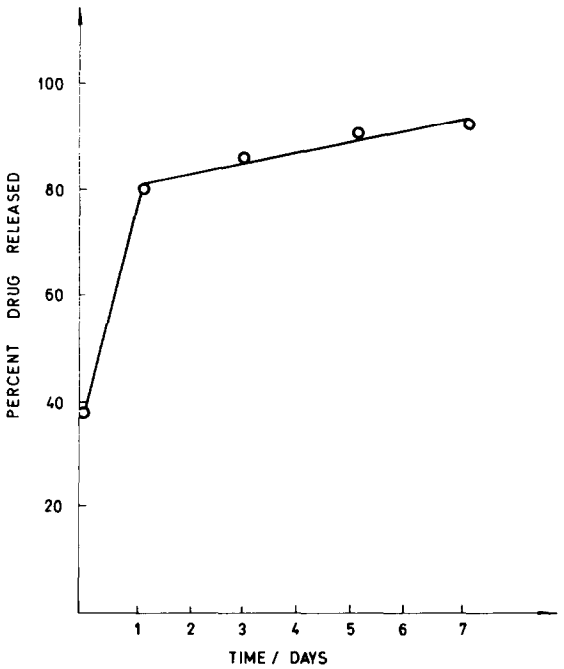

Fig. 3. In vitro release of acetylsalicylic acid (Method II) during the total period of investigation of glycosylation.

because of its reproducibility and the technical simplicity that is needed to follow glycosylation over a period of several days. Table 1 lists the concentrations of glycosylated proteins during 1 week of incubation of mixtures A and B. The results clearly demonstrate the inhibitory effect of acetylsalicylic acid on protein glycosylation. It should be noted that no difference in glycosylation was observed with the sample containing poly(lactic acid) but not poly(lactic acid) microcapsules. This observation excludes the possibility of interaction of the polymer matrix on glycosylation. The effect was enhanced when microencapsulated drug was applied instead of the unencapsulated form. If the results are expressed as a percentage of the 0-day values (100%) (Figs. 4 and 5), this trend is even more obvious. The same situation was found for both mixtures A and B containing different amounts of glucose and acetylsalicylic acid, the only difference being the concentrations of glycosylated proteins which were higher for mixture B. The results obtained for acetylsalicylic acid are basically in agreement with previous findings by other authors (Hawkins et al. 1969; Day et al. 1979a, b; Rendell et al. 1986), however, the enhancement of inhibition obtained

TABLE 1

Glycosylation of serum proteins determined by the fructosamine test [a]

Sample	Time (day)			
	0	1	4	7
A1	3.2	4.4	6.9	8.2
A2	3.3	4.0	6.0	7.6
A3	3.4	4.0	5.3	6.6
B1	3.7	5.5	9.6	9.9
B2	3.5	4.4	7.3	7.8
B3	4.0	4.0	6.0	9.1

[a] Expressed as concentration (mmol/l) of 1-deoxy-1-morpholino-fructose (DMF).

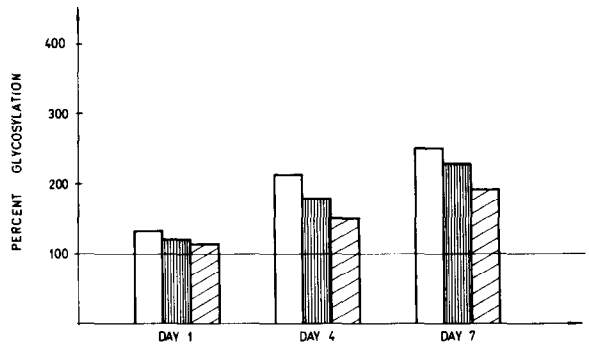

Fig. 4. Glycosylation of serum proteins expressed as percent of 0-day values: □, A1; ▥, A2; ▨, A3.

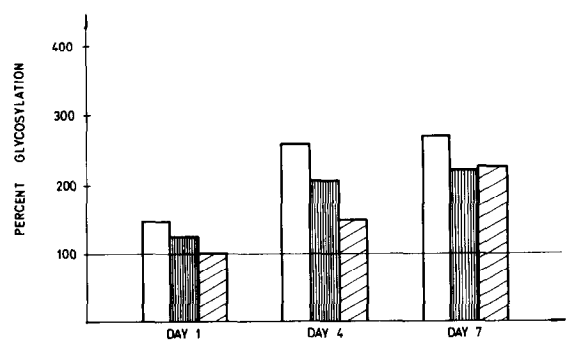

Fig. 5. Glycosylation of serum proteins expressed as percent of 0-day values: □, B1; ▥, B2; ▨, B3.

with the microencapsulated drug was not previously studied.

Two possible explanations for this phenomenon can be proposed. It has been demonstrated by Rendell et al. (1986) that acetylsalicylic acid inhibits glycosylation by prior acetylation of the amino groups on the protein, whereas salicylic acid, obtained by rapid hydrolysis, is not an acetylating agent. Rapid hydrolysis of acetylsalicylic acid to salicylic acid with a $t_{1/2}$ value of about 23 min (Castello and Green, 1982) would suggest that the acetylsalicylic acid concentration available for acetylation is rapidly decreased in solution. In the case of microcapsules, their matrix structure protects acetylsalicylic acid to a certain degree and the process of hydrolysis to salicylic acid is delayed. Therefore, high drug concentrations are available to acetylate proteins. The second hypothesis takes into account the concentration gradient of glucose from the bulk solutions to the core of a microcapsule. From microcapsule research it is known that, due to their permeability, microcapsules can be used to remove various substances of low and intermediate molecular weight from the surrounding medium. If a fraction of glucose enters microcapsules, then its concentration in the bulk solution will be diminished as well as glycosylation of proteins.

Experiments were performed with only one type of polymer material and a single type of capsule whose particle size distribution was rather broad. Also, only two levels of glucose and drug were used. Evidently, the present investigation should be extended to give less speculative answers. Further studies involving microcapsules that are better defined and studied in an in vivo situation are currently underway. It appears that the present investigation offers at least a theoretical possibility of increasing inhibition of protein glycosylation by using a novel approach.

References

Alberti, K.G.M.M. and Press, C.M., The biochemistry of complications of diabetes mellitus. In Keen H. and Jarret, J. (Eds.), *Complications of Diabetes*, 2nd Edn, Edward Arnold, London, 1982, pp. 231–271.

Castello, P.B. and Green, F.A., Aspirin survival in human blood modulated by the concentration of erythrocytes. *Arthritis Rheum.*, 25 (1982) 550–555.

Day, J.F., Thorpe, S.R. and Baynes, J.W., Nonenzymatically glycosylated albumin. In vitro preparation and isolation from normal human serum. *J. Biol. Chem.*, 254 (1979a) 595–597.

Day, J.F., Thornburg, R.W., Thorpe, S.R. and Baynes, J.W., Nonenzymatic glycosylation of rat albumin. Studies in vitro and in vivo. *J. Biol. Chem.*, 254 (1979b) 394–400.

Deasy, P.B., *Microencapsulation and Related Drug Processes*, Dekker, New York, 1984.

Hawkins, D., Pinckard, R.N., Crawford, J.P. and Farr, R.S., Structural changes in human serum albumin induced by ingestion of acetylsalicylic acid. *J. Clin. Invest.*, 48 (1969) 536–542.

Hopper, A.H., Tindall, H. and Davies, J.A., Effect of aspirin on glucose tolerance and insulin levels in patients with type 2 diabetes mellitus. *Thromb. Haemostasis*, 54 (1985) 895.

Jalšenjak, I., In vitro release from microcapsules and microspheres. In Donbrow, M. (Ed.), *Microcapsules in Medicine and Pharmacy*, CRC Press, Boca Raton, 1990, in press.

Jerntorp, P., Sundkirst, G., Fex, G. and Jepson, J.-O., Clinical utility of serum fructosamine in diabetes mellitus compared with hemoglobin A_{1C}. *Clin Chim. Acta*, 175 (1988) 135–142.

Jick, H., Effects of aspirin and acetaminophen in gastrointestinal hemorrhage. *Arch. Intern. Med.*, 141 (1981) 316–321.

Johnson, R.N., Metcalf, P.A. and Baker, J.R., Fructosamine: a new approach to the estimation of serum glycosylprotein. An index of diabetic control. *Clin. Chim. Acta*, 127 (1982) 87–95.

Kennedy, L. and Lyons, T.J., Non-enzymatic glycosylation. *Br. Med. Bull.*, 45 (1989) 174–190.

Koskinen, P., Irjala, K., Viikari, J., Panula-Ontlo, R. and Matikainen, M.T., Serum fructosamine in the assessment of glycaemia control in diabetes mellitus. *Scand. J. Clin. Lab. Invest.*, 47 (1987) 285–292.

Phillipou, G., Scaborn, C.J. and Phillips, P.J., Re-evaluation of the fructosamine reaction. *Clin. Chem.*, 34 (1988) 1561–1564.

Reid, J., MacDougall, A.J. and Andrews, M.M., Aspirin and diabetes mellitus. *Br. Med. J.*, 2 (1957) 1071–1074.

Rendell, M., Nierenberg, J., Brannar, C., Valentine, J.L., Stephen, P.M., Dodds, S., Mercer, P., Smith, P.K. and Walder, J., Inhibition of glycation of albumin and hemoglobin by acetylation in vitro and in vivo. *J. Lab. Clin. Med.*, 108 (1986) 286–293.

Richterich, R. and Colombo, Y.P., *Clinical Chemistry*, Wiley, New York, 1984.

Winocour, P.H., Bhatnagar, D., Reed, P. and Dhar, H., Does the measurement of serum fructosamine accurately reflect levels of glycated albumin in insulin-dependent diabetes? *Ann. Clin. Biochem.*, 24 (1987) 47–52.