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Stabilisation of a protein prodrug formulation: a novel approach

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Summary

APSAC (anisoylated plasminogen streptokinase activator complex) is a prodrug of the fibrinolytic lys-plasminogen streptokinase complex. Is is prepared by acylation of the serine hydroxyl group in the active centre of plasminogen, and is formulated as a freeze-dried powder for reconstitution and intravenous administration. The acyl group is labile in solution, and to a lesser extent also in the lyophilised state. Here we report a method for stabilising the prodrug by formulation with a water-soluble acylating agent. On reconstitution, any free hydroxyl groups formed during storage are rapidly reacylated while essentially full acylation is maintained for the required period in solution.

Introduction

The use of prodrugs to improve pharmacokinetic and pharmaceutical properties of drug substances has been reviewed extensively (Bundgaard, 1985, 1991; Bodor and Kaminsky, 1987; Waller and George, 1989).

Esterification of hydroxyl groups, or *N*-acyl derivatisation of amines, are common ways of altering solubility and bioavailability; hydrolysis of the esters and amides to regenerate the parent drug in vivo may occur either enzymatically or spontaneously. A common problem, however, is that rates of spontaneous hydrolysis in aqueous solution are sufficiently great to be a significant factor in the stability of the product.

Various solutions have been derived to this problem, with varying degrees of success. For example, the prodrug may be formulated at the pH where hydrolysis rates are at a minimum, often around pH 4–5 (Repta et al., 1975; Bundgaard et al., 1984, 1988; Bundgaard and Falch, 1985a). The stabilization thus achieved has not always proved adequate.

The acyl group may sometimes be modified, or additional groups in the parent drug may be derivatised to improve stability (Bundgaard and Falch, 1985b; Bundgaard et al., 1986, 1989; Jensen et al., 1990) but this will generally also effect solubility, absorption and in-vivo hydrolysis rates.

A third solution is to formulate the prodrug as a dry powder for reconstitution (Repta et al., 1975; Bundgaard et al., 1984).

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APSAC (BRL26921, EMINASE *) is a fibrinolytic prodrug (Smith, 1983; Waller and George, 1989), prepared by acylation of the hydroxyl group on a serine residue in the active centre of the parent compound, the lys-plasminogen-streptokinase complex. It is administered by i.v. injection. The prodrug is cleared from the bloodstream much more slowly than the plasminogen-streptokinase complex which is formed in vivo on administration of streptokinase, and in addition it shows greater stability to proteolytic degradation in plasma (Fears et al., 1987).

In APSAC, the chemical structure of the acyl group determines the rate of in vivo deacylation, which is crucial to the speed and duration of action. APSAC is known to deacylate under physiological conditions with a half-life of around 120 min at 37°C. (Hibbs et al., 1989).

The range over which the pH of the formulation can be adjusted is limited by considerations of solubility and stability of the complex. Lyophilisation contributes greatly to stabilisation, but for this compound is not adequate to ensure an acceptable shelf-life. In order further to stabilise the formulation, a different approach was necessary.

This manuscript describes how this was achieved by including in the formulation a small quantity of the water-soluble acylating agent *p*-amidinophenyl-*p'*-methoxybenzoate, the acylating agent which is also used in the synthesis of APSAC.

Materials and Methods

Materials

The preparation of APSAC (Smith et al., 1982) and of *p*-amidinophenyl-*p'*-methoxybenzoate hydrochloride (*p*-amidinophenyl-*p'* anisate · HCl or APAN) (Smith, 1980) have both been described previously. APAN was used by appropriate dilution from a stock solution of 20 mM in dimethylsulphoxide. The chromogenic substrate S-2251 (H-D-Val-Leu-lys-*p*-nitroanilide · HCl), which is specifically cleaved by plasmin-like enzymes to generate *p*-nitroaniline, was obtained from Kabi-Vitrum, Stockholm, Sweden.

Gel filtration

To remove APAN from an APSAC solution, a 500 μl aliquot was applied to a prepared column of Sephadex G-25M ('PD-10', Pharmacia LKB Biotechnology, Uppsala, Sweden) which had been pre-equilibrated with a buffer containing L-lysine · HCl (25 mM), D-mannitol (1% w/v) and 6-aminohexanoic acid (1 mM) at pH 7.6. The column was eluted with 4.1 ml of the same buffer, the first 1.6 ml being discarded.

Determination of activator activity

S-2251 was dissolved to 2.5 mM in a pH 8.4 buffer containing triethanolamine · HCl (0.1 M) and sodium azide (0.05% w/v), and equilibrated to 25°C. To 600 μl of this solution, placed in a semi-micro cuvette, 10 μl of APSAC solution was added. The rate of increase in absorbance at 405 nm was then immediately measured over a 1–2 min period in a double-beam UV/Vis spectrophotometer fitted with a thermostatted cell-holder (Perkin-Elmer Lambda 3 or Lambda 5). The activity thus measured was expressed as a percentage of the maximum activity measured after complete deacylation of all the APSAC in the solution. Complete deacylation was achieved either by incubation for 6 h at 25°C in a pH 8.6 buffer containing Tris (0.1 M), glycerol (20% v/v), Tween 20 1% v/v) and sodium chloride (0.9% w/v), or alternatively for 2 h at 37°C in a pH 9.0 buffer containing Tris (12.5 mM), L-lysine · HCl (2.5 mM), glycerol (50% v/v) and sodium chloride (0.45% w/v).

Determination of APAN concentration by HPLC

Hydrolysis of APAN in APSAC solutions was stopped by addition of 20% v/v of 0.2 M sodium acetate buffer at pH 4.5. Protein was then removed by precipitation with two volumes of methanol. After 10 min in an ice bath, the precipitate was separated by brief centrifugation. The supernatant (10 μl) was injected into a Spherisorb 5 ODS column (25 cm × 4.6 mm) and eluted isocratically with a mixture of 1 volume of 0.05 M

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sodium acetate buffer at pH 4.5 and 4 volumes of methanol, at a flow rate of 1.5 ml/min. Peaks were detected by UV absorbance at 260 nm.

Results and Discussion

The deacylation of APSAC was studied over 22 h at 25°C and pH 7.6. Residual APAN from the manufacturing process was first removed by gel filtration, and the appearance of activity of the free (deacylated) activator complex was monitored with time. Curve 1 in Fig. 1 shows the recovery of activity with time. It follows approximately first-order kinetics, with a half-life under these conditions of approx. 8 h, less than 1 h being required to reach 10% free activator complex. This rate of hydrolysis was not regarded as acceptable for Eminase, a commercial formulation of APSAC, which had a design specification of less than 3% free complex activity on injection.

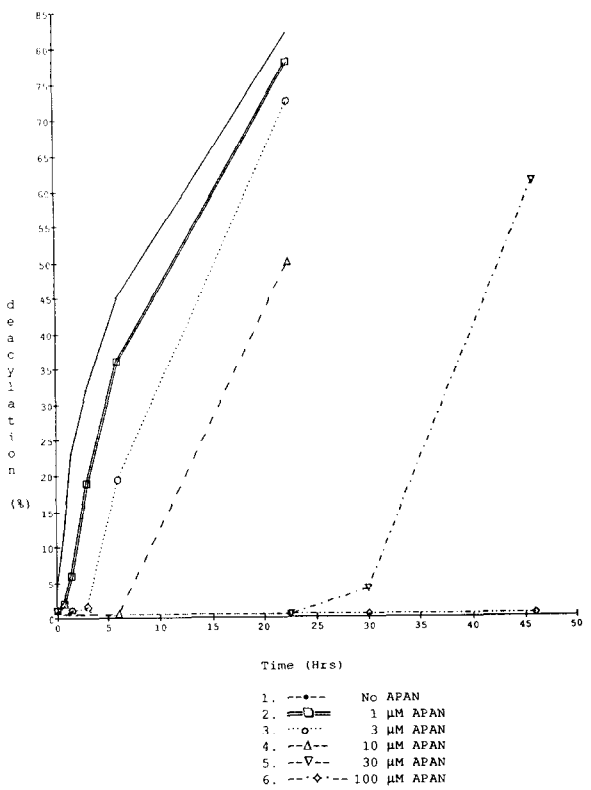

Fig. 1. Effect of APAN on deacylation of APSAC.

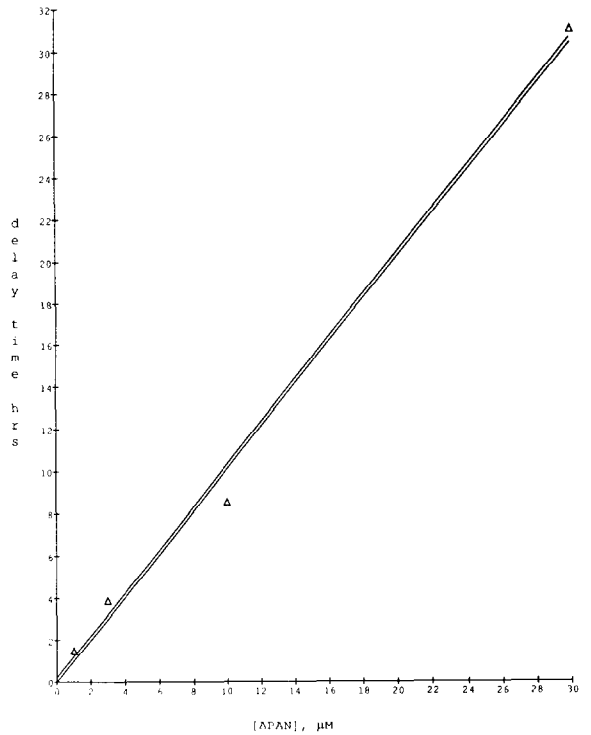

Fig. 2. Effect of added APAN on delay in reaching 10% deacylation.

The scope for stability improvements by manipulation of the formulation pH proved to be limited with APSAC: its solubility decreases to unacceptable levels at lower pH values, while at higher pH deacylation proceeds more rapidly. Lyophilisation also proved to be inadequate in itself to provide long term stability of the acyl group; one early experimental lyophilised formulation reached 10% free activator complex content after 8 days storage at 30°C.

It was noted, however, that residual APAN from the manufacturing process could have a marked stabilising effect. Curves 2–6 in Fig. 1 show the effect of adding varying amounts of APAN to a solution of APSAC in formulation buffer. Increasing amounts of APAN delayed the onset of deacylation for lengths of time which were found to be directly proportional to the amount added. Fig. 2 shows this relationship.

It may be concluded that low levels of APAN in solution re-acylate de-acylated APSAC very efficiently, maintaining a very low level of free activator complex until the APAN itself is largely consumed. This situation is analogous to that described for the 'titration' of thrombin using ethyl-*p*-guanidinobenzoate. (Chase and Shaw, 1969). Based on this work, the overall reactions can be described by:

$$\text{SK-PG} + \text{APAN} \overset{K_S}{\longleftrightarrow} \text{SK-PG.APAN} \xrightarrow{k_2} \text{AP}$$

$$+ \text{APSAC} \xrightarrow{k_3} \text{SK-PG} + \text{AN}$$

such that the free active centre (SK-PG) released by hydrolysis of the *p'*-anisoyl group is returned to the pool of free enzyme for reacylation. Taking this analogy further to include a second equation derived by Chase and Shaw (1969), at steady state the ratio of total activator complex (SK-PG plus APSAC) concentration over acyl-activator complex (APSAC) concentration may be described by

$$[\text{SK-PG} + \text{APSAC}]/[\text{APSAC}]$$

$$= 1 + k_3/k_2 + k_3K_s/k_2[\text{APAN}_0]$$

where $[\text{APAN}_0]$ represents the starting concentration of APAN. In this case, the ratio remains very close to 1 ([SK-PG] is very small compared to [APSAC]), as would be expected when $k_2 \gg k_3$. Smith et al. (1982) designed APAN with this in mind. Assuming temperature and pH are held constant, k_2 and k_3 are invariant and the only means of altering the concentration of SK-PG is to adjust the concentration of APAN. As the concentration is progressively increased above the concentration of SK-PG and APSAC, further changes in levels of free enzyme are negligible, and the time delays before onset of noticeable levels of SK-PG are progressively increased.

This is illustrated in Table 1. APSAC solution in buffer adjusted to pH 8.0 (at around 50 μM total activator complex concentration), with APAN added to a final concentration of 145 μM was kept at 25°C for 10 h. At 1 h intervals, APAN concentration and free activator complex content were measured. APAN was almost totally consumed before significant levels of free activator complex started to appear.

TABLE 1

Loss of APAN and appearance of free activator complex in APSAC solution at pH 8.0, 25°C

Time (h)	[APAN] (μM)	Free activator complex (%)
0	145	0.2
1	122	0.2
2	101	0.2
3	80	0.2
4	65	0.2
5	56	0.2
6	44	0.3
7	34	0.3
8	26	0.3
9	18	0.4
10	8	0.8

It thus seemed that incorporation of APAN into the formulation could stabilise the product. However, significant levels of free active centre can be formed in the lyophilised product, and reacylation does not occur until the material is reconstituted into an aqueous solution. It was necessary to confirm that reacylation would occur sufficiently rapidly. To study the feasibility of reacylation on reconstitution and to estimate the approximate amount of APAN required, a lyophilised formulation of APSAC containing only a small residue of APAN was stored under conditions expected to generate a significant amount of free activator complex. Three equal portions of the formulation were reconstituted in different diluents to give a total complex concentration of about 50 μM, the first portion in water (HPLC analysis showed it to contain 8 μM APAN as a residue from the manufacturing process), the second in 30 μM APAN in water, and the third in 100 μM APAN solution. The free activator content was monitored over 8 min at 25°C.

As shown in Table 2, under these conditions, APAN in the solution used for reconstitution re-acylated free activator complex rapidly, at a rate which was proportional to the amount of APAN added. Use of this method of stabilisation of the marketed product is not feasible, however, because of the limited long-term stability of

TABLE 2

Effect of APAN added at reconstitution on reacylation of free activator formed during storage in the freeze-dried state

Time, after reconstitution (min)	Free activator (%)		
	In H_2O	In 30 μM APAN	In 100 μM APAN
0.5	3.9	2.2	1.1
1	4.3	1.6	0.5
2	3.6	0.6	0.2
4	4.3	0.2	0.1
8	3.0	0.1	0.1

APAN itself in aqueous solution; incorporation of APAN into the lyophilised product appeared to be the logical alternative.

To investigate the effectiveness of co-lyophilised APAN, an APSAC solution of 50 μM activator complex concentration was prepared. This was divided into three portions, and APAN was added to give final concentrations of around 75, 90 and 115 μM respectively. The solutions were then aliquotted into vials, lyophilised and stoppered. Free activator levels, measured over a period of 30 min after reconstitution at 25°C, were determined on vials immediately after lyophilisation and after 2 and 4 months storage at 30°C. The results are shown in Table 3. In all cases, reacylation takes place within a few minutes of reconstitution to reach the steady state level of around 0.2% free activator complex.

TABLE 3

Effect of co-lyophilised APAN on re-acylation after various periods of storage in the freeze-dried state

Storage time (in dry state) at 30°C	Time in solution (min)	Free activator (%)		
		In 75 μM APAN	In 90 μM APAN	In 115 μM APAN
Initial	0.5	0.2	0.2	0.2
	1	0.2	0.2	0.1
	2	0.2	0.2	0.2
	7	0.1	0.2	0.2
	13	0.1	0.2	0.1
	30	0.2	0.2	0.1
2 months	0.5	3.1	2.7	2.4
	1	1.5	1.6	1.2
	2	0.6	0.9	0.6
	7	0.3	0.2	0.2
	13	0.2	0.2	0.2
	30	0.2	0.2	0.2
4 months	0.5	4.0	3.6	2.6
	1	2.1	2.3	2.3
	2	1.2	0.9	0.9
	7	0.5	0.2	0.2
	13	0.3	0.2	0.2
	30	0.2	0.2	0.2

These findings have contributed toward optimisation of the formulation of APSAC, which, as Eminase, now has a 3 year shelf-life at refrigerated temperatures, is stable for up to 12 months at ambient temperatures and is stable for over 1 h in solution following reconstitution in water.

Use of APAN in this way in the product has been shown to be toxicologically acceptable.

Conclusions

The prodrug APSAC was found to convert to its active thrombolytic form on storage. Although most pronounced in solution, this conversion can also occur in lyophilised formulations. Following incorporation of APAN into the formulation, any prodrug which has hydrolysed is rapidly reacylated when the product is reconstituted in aqueous solution. This novel approach has provided an acceptable stability profile for the marketed product.

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