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FORMULATION STUDIES ON [2-amino-5-bromo-6-phenyl-4(3)-pyrimidinone] (ABPP), AN INTERFERON INDUCER, ANTI-CANCEROGENIC AGENT

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ABSTRACT

The apparent solubility of ABPP in aqueous solutions suitable for intravenous use was increased by cosolvent systems.

The solubility of ABPP was determined as a function of pH and concentration of NaOH, NaCl, Na_2CO_3 and co-solvents. Partition coefficient of ABPP in Octanol/water systems were also determined at various pH values. Solubility data and spectrophotometric methods were used to determine the pKa values. The solubility of the compound increased at lower and higher pHs with increasing NaOH and Na_2CO_3 concentrations and increasing PEG 400, Propylene glycol, dimethylformamide and dimethylacetamide concentrations but decreased with the introduction of NaCl to the solution. The apparent partition coefficient of ABPP showed similar but reversed dependence to the pH of the buffer system.

From these results, suitable formulations for use as parenteral solutions are proposed. The increase in the apparent aqueous solubility of ABPP in such formulations may increase up to 800-fold, depending upon the pH and co-solvent concentrations. The compound was found to be stable for at least 6 months (experimental period) under normal shelf conditions when formulated for i.v. administration. When the suggested formulation injected intravenously into rabbits, at three different dose levels, the elimination half life of ABPP appeared not be be dose dependent although data showed a straight line relationship between the area under the curve and dose and Cpmax and dose.

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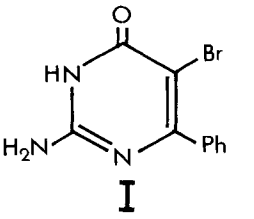
INTRODUCTION

2-Amino-5-bromo-6-phenyl-4(3)-pyrimidinone (ABPP, I) induces endogenous interferon (IFN) in several animal species (1,2) and has preclinical antiviral (3), antitumour and immunostimulatory activities (4,5). Initial Phase I trials revealed poor bioavailability in man when administered as an oral dose, although objective antitumour responses occurred (6). Further clinical trials have been hampered by the lack of a suitable injectable form of the drug due to the very low solubility ($\sim$39 microgram/ml). This study was undertaken to develop a suitable intravenous formulation of ABPP and by this means obviate the problems associated with the GI absorption of the nearly insoluble drug.



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EXPERIMENTAL

Materials - ABPP and ABmFBP (2-amino-5-bromo-6-(3-fluorophenyl)-4(3H)-pyrimidinone) were supplied by the Upjohn Company and used as received. All the solvents used were of spectroscopic grade and other reagents were of analytical grade.

Equipment - The pH values of aqueous buffer solutions were measured with a pH meter with an accuracy of 0.01 pH unit (Radiometer pH M64 Research pH meter). UV absorption measurements relevant to the determination of partition coefficients and the pKa determination of ABPP by the UV method were made using a spectrophotometer (Cecil CE292 UV spectrophotometer series 2). High-performance liquid chromatography (HPLC) was undertaken with a Gilson Model 802 Monometric solvent delivery module equipped with a CECIL CF 202 variable-wave-length UV detector, operated at 290nm with a sensitivity of 0.2 AUFS and a Rheodyne 7125 injection valve with a 20 µl loop. A reversed-phase column, 10cm x 4.6 mm, packed with Spherisorb-ODS (5 µm) was used. HPLC was employed for the determination of ABPP in plasma and for stability experiments.

Solubility Determinations - The solubility of ABPP in different buffers as a function of pH, in NaOH, Na_2CO_3 and NaCl solutions concentration and in different co-solvent systems was determined in the following manner. An exact quantity of ABPP (ca 2-3g) was added into a flask and 10 ml of the appropriate medium was added. The flasks were mechanically shaken and then suspended in a constant temperature bath maintained at 25°C (±0.1) for several days. The attainment of equilibrium was verified by repetitive measurements after several additional days. Aliquots of saturated ABPP solutions were filtered through a 0.2 m cellulose nitrate micropore filter and diluted quantitatively

to give an absorbance reading within the spectrophotometric range. Analysis carried out at 310nm in triplicates and the results averaged.

Data obtained from the solubility determinations were used to estimate pKa values.

Partition coefficient Determinations - Five milliliters of 7.5×10^{-4}M ABPP in buffer-saturated Octanol was individually added onto 25 milliliter of Octanol saturated buffer (pH 2-12) in a screw-capped vial.

The mixtures were then incubated for 48hr in a temperature-controlled water-bath (± 0.1C°) with occasional vigorous agitation with a whirlmixer. After equilibrium was reached the flasks were allowed to stand to allow the layers to separate. Aliquots of the organic phase were withdrawn subjected to the analysis using saturated octanol as reference. The pH of the aqueous phase was measured immediately.

Preparation of Buffer Solutions

Britton-Robinson (pH 1.81-11.98) buffer solutions (7) were prepared by mixing suitable amount of sodium hydroxide, acetic acid, phosphoric acid and boric acid in water and the ionic strengths were adjusted to 0.5M using potassium chloride. The ionic strength of the Britton-Robinson buffers were kept constant throughout. The pH values of the solutions were checked at 22°C with a pH meter which had been calibrated at the same temperature using standard buffers. Delory-King Buffer (pH 9.8 - 10.60) (8) was also prepared from the 0.2 molar anhydrous sodium carbonate and 0.2 molar sodium carbonate stock solutions.

PKa Determination by Spectrophotometric Method

The Spectrophotometric method (9) was used to measure the pKa of ABPP at 3 independent analytical wavelengths; 210, 270 and 310 nm. Fourteen different ABPP solutions (0.001mg/ml) were prepared with pH values ranging from 2-11. These solutions were adjusted to the desired pH by adding 1M HCl or 1M NaOH. The pH of each solution was measured prior to the absorbance reading. The blank was the solvent without the sample. The spectra of 14 solutions were thus obtained and the pKa was calculated using the expression:

$$pKa = pH + \log \frac{dm - d}{d - di} \qquad (1)$$

Where dm = absorbance of the unionized species, di = absorbance of the ionized species and d = absorbance at that pH.

Stability Tests -

a) 20mg ABPP was dissolved in 250ml 10% DMF in Britton-Robinson buffer. The final pH was 10.34. The solution was maintained at 37°C in a water-bath for a period of 2 months, during which time samples were withdrawn at intervals and analysed by HPLC.

b) 10mg ABPP was dissolved in 100ml 10% DMA in 0.2M sodium carbonate with pH 10.4. The solution was left to stand at room temperature for 8 months. Samples were withdrawn at intervals and chromatographed.

Pharmacokinetic Studies -

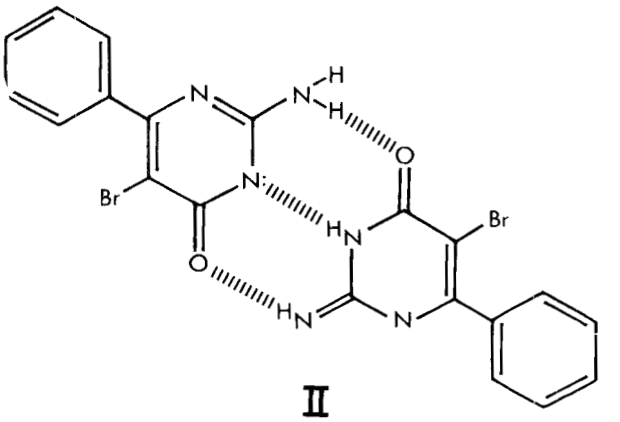
White New Zealand Rabbits (3-4kg) were used for in vivo studies together with a formulation of ABPP suitable for intravenous injection containing (20mg/ml) in 10% $^{v}/_{v}$ DMA solution at 10.4 pH prepared and sterilized by autoclaving at 121°C 15 lbs^{-2} for 20 minutes. Four rabbits were weighed and the dose for each rabbit was calculated on the weight basis (5, 10 & 25mg/kg) and slowly injected into the marginal ear vein. Blood samples (3 ml) were collected from the marginal vein of the other ear at the time intervals up to 24 hrs. After the extraction procedure the samples analysed by HPLC method. For extraction, the samples were centrifuged to separate the serum, a quantity of which was transferred to another centrifuge tube. 100 microliters of internal standard (ABmFPP) solution (10 µg/ml) was added and the pH was adjusted to 3 with 1M HCl. The samples were mixed and 2.5ml ethylacetate were added. After mixing the tubes were then centrifuged at 3500 rpm for 20 min, and ethyl acetate layer separated. The ethyl acetate containing the ABPP and internal standard was evaporated to dryness, the residue was dissolved in 1ml methanol and chromatographed.

RESULTS & DISCUSSION

a) Solubility in aqueus systems:

The Solubility of ABPP in different systems at 25°C is shown in Figs 1, 2, 3. When the effect of pH on the solubility of ABPP was investigated (Fig 1), it was shown that the solubility profile of ABPP goes through a minimum as the solution pH increases from 2 to 12.

Although the ABPP molecule possesses polar groups it is poorly soluble in II water presumably due to the formation of dimers (7) at pH between 4 and 7.5 (II). However, by increasing or decreasing pH ionisation occurs which results in increasing solubility due to reduced molecular interactions and better solvation of the charged species. This behaviour confirms the amphoteric nature of ABPP.



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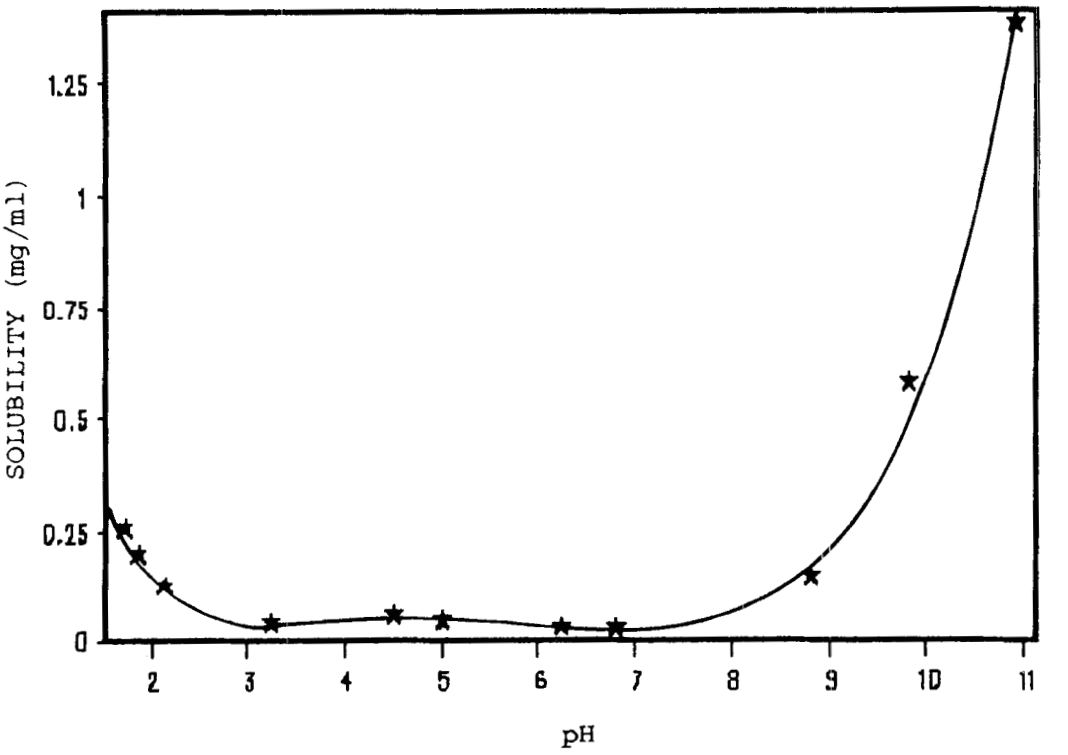

FIGURE 1

SOLUBILITY OF ABPP IN BRITTON-ROBINSON BUFFERS

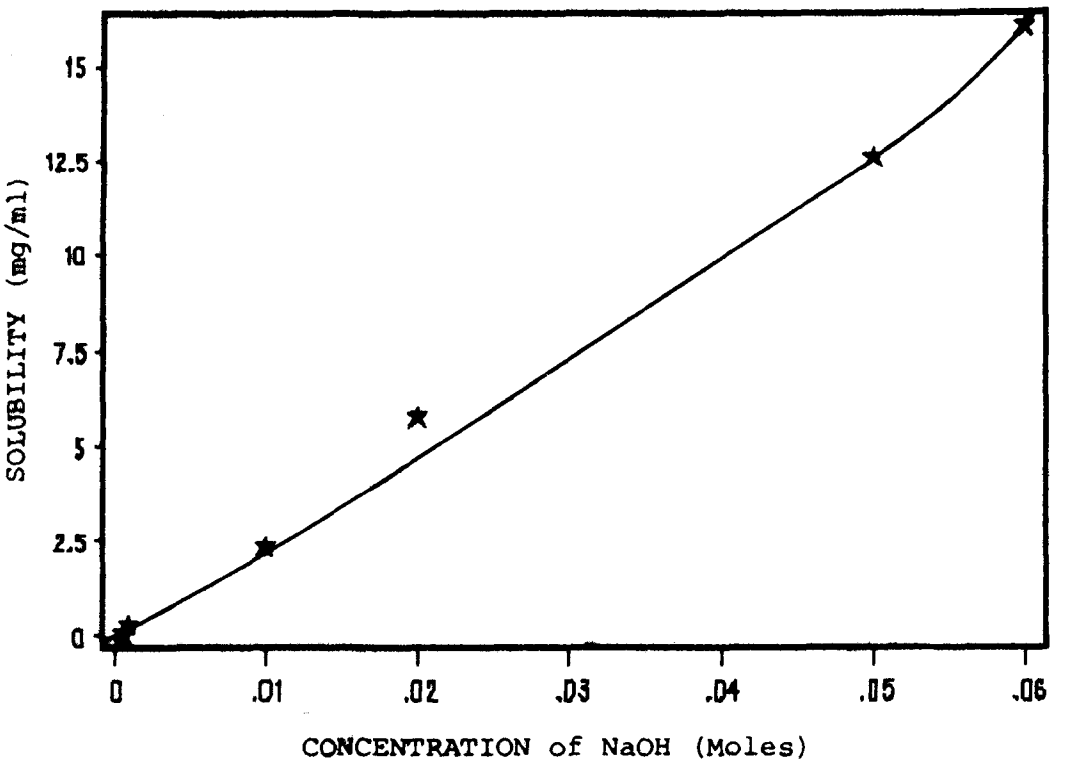

FIGURE 2

SOLUBILITY of ABPP in DIFFERENT CONCENTRATIONS of NaOH SOLUTIONS

In order to extend the solubilities in base, the effects of sodium hydroxide solutions on ABPP were also studied. The solubility of ABPP as a function of sodium hydroxide concentration is shown in Fig 2. It is obvious that the apparent solubility of ABPP is greatly enhanced by the presence of added base.

Since higher pH media gave promising solubility results, the solubility characteristics of ABPP at pH >7 were further studied using carbonate buffers (pH 9.87 and 10.58). Here, the solubility of ABPP was 5 to 10 times higher than those obtained at the comparable pH values with either Britton-Robinson buffer or aqueous sodium hyroxide Table 1.

This behaviour may be due to complexation involving carbonate - bicarbonate ions which reduces the intermolecular attraction of ABPP molecules.

TABLE 1. Solubility of ABPP in Different Aqueous Systems.

pH	Solubility (microgram/ml)		
	Delory-King Buffer	Britton-Robinson Buffer	Sodium Hydroxide
9.9	1580	423	74.2
10.6	4492	1284	300.5

Although the solubility could be increased with sodium hydroxide to about 200 mg/ml at pH 13.0 this pH is too high to be of use as an intravenous vehicle. In contrast the solubility in sodium hydroxide at pH 10.72 was only 0.3 mg/ml and rather low for adequate delivery. In an attempt to improve this behaviour the effect of sodium carbonate on the solubility of ABPP was also studied (Fig 3).

Sodium carbonate increased the pH further than was possible with carbonate:bicarbonate buffer and also increased the solubility to about 10 mg/ml at pH 11.49 (0.2M Na_2CO_3). This appeared a fair compromise between solubility and pH. The highest pH of any intravenous injection commercially available appears to be that of phenytoin which is pH 12. It would be highly inadvisable to exceed this limit.

Since many products for intravenous use are administered as infusions and are routinely diluted with parenteral vehicles, the addition of sodium chloride solutions to the ABPP solutions in Delory-King buffer was examined and summarised in Table 2.

When the effect of a concentration range of (0 - 6% $^{w}/v$) NaCl in Delory-King buffer was investigated it was found that the solubility of ABPP was decreasing as the concentration of NaCl was increasing. This effect could be due to the salting out effect of sodium chloride.

b) Solubility of ABPP in Cosolvent Systems.

The cosolvents used were propylene glycol (PG), polythylene glycol 400 (PEG 400), N, N - Dimethylacetamide (DMA) and N,N-Dimethylformide (DMF). The

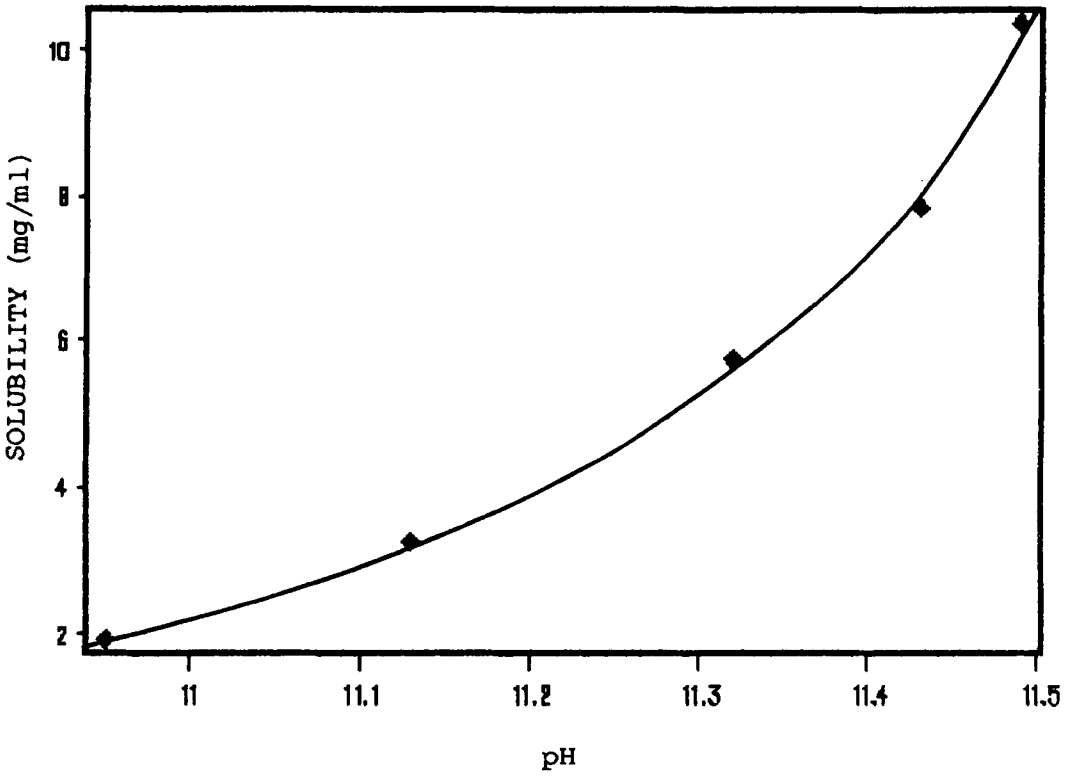

FIGURE 3

SOLUBILITY of ABPP in SODIUM CARBONATE SOLUTIONS

TABLE 2

The Effect of Sodium Chloride Concentration on the Solubility of ABPP in Delory-King Buffer at pH 10.46, at 25°C

Concentration of NaCl (% w/v)	Solubility (microgram/ml)
0	4492.0
0.6	4080.0
1.2	3774.4
3	3179.7
6	2464.9

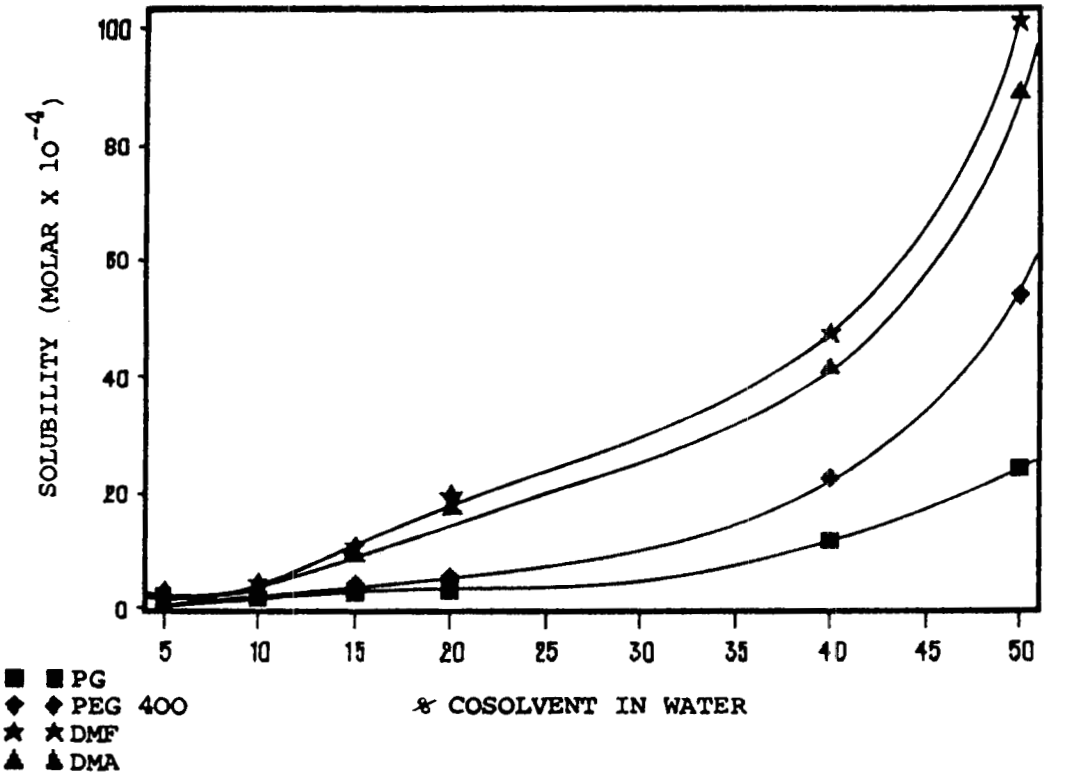

FIGURE 4

SOLUBILITY OF ABPP IN COSOLVENTS

effects of concentrations of these cosolvents on the solubility of ABPP are illustrated in Fig 4. Each cosolvent increased the solubility of ABPP. However, DMF and DMA, were similar in effect and were far superior to PEG 400 and PG, solubilizing approximately 25mg/ml of drug at a 50% ($^{V}/v$) cosolvent composition.

The plots of log solubility of ABPP against different cosolvents in water showed straight line relationships over the range 0-50% ($^{V}/v$) cosolvent (Table 3). Since % cosolvent is proportional to volume fraction compliance with the solubility equation (8) is shown

$$\log Sm = \log Sw + \sigma fc \qquad (2)$$

where Sm = molar concentration of solute in solvent mixture.
Sw = molar solubility of solute in water

TABLE 3

Effect of Different Cosolvents on the Solubility of ABPP in Water at 25°C

Cosolvent	log Sw	σ	r
PG	- 3.945	0.02591	0.997
PEG 400	- 3.945	0.0316	0.999
DMF	- 3.672	0.03369	0.997
DMA	- 3.743	0.0342	0.999

Mean calculated water solubility = 39.9 mcg/ml
Mean value for water solubility from direct measurement = 40 mcg/ml

σ = solubility power of the cosolvent
fc = volume fraction of cosolvent

Hence Sw can be found by extrapolation of the curve to 0% cosolvent and can be calculated (Table 3) for each cosolvent by the least squares method of linear regression from the slope. Through these calculations the mean solubility in water was calculated and found as 1.500×10^{-4}M (SD 0± 1214) = 39.9 mcg/ml. Experimentally the water solubility of ABPP was found to be 40 mcg/ml which is in agreement with the above result. Graphical presentation of log solubility or log $\frac{S_w}{S_m}$ of ABPP against either dielectric constant (ε) or resiprocal dieletric constant plots also produced linear relationships (r = 0.997 and r = 0.9996 respectively).

Although 100% DMSO also showed high saturated solubility levels, precipitation occurred both on standing, after 4 hrs, and also when was added to normal saline, 5% Dextrose or plasma substitute. On the other hand the DMA based solution did not precipitate at any dilution or on standing.

When both DMA and PEG (400) were tried in conjunction with 0.2M Na_2CO_3, the solubility of ABPP was increased considerably to 22 mg/ml and 14 mg/ml respectively at 10% (^{v}/v) cosolvent. This result is presumably a combined effect of solvents and pH. Possibly;
a) by adjusting the polarity of the solvent to a more favourable value

TABLE 4

Effect of PEG 400 and DMA Concentration on the Solubility of ABPP in 0.2M Sodium Carbonate.

% (v/v) Cosolvent in 0.2M Na_2CO_3	Concentration of ABPP (mg/ml)
Polyethylene Glycol (400)	
0	10.4
5	11.453
10	14.682
15	19.250
Dimethylacetamide	
0	10.4
5	14.053
10	21.621
20	25.212
30	28.438

b) by changing the pKa.
The result of this experiment is shown in Table 4.

pKa Determinations:

a) Solubility method - the results obtained from solubility of ABPP at different pH values were used to calculate the pKa values of ABPP by employing the equations;

$$pKa = pH - \log [(S/So) - 1] \text{ for acids} \quad (3)$$
$$pKa = pH + \log [(S/So) - 1] \text{ for bases} \quad (4)$$

where So = solubility of neutral molecular species.
S = solubility of compound at that pH.

By rearranging equation 3;

$$S = So + \frac{kaSo}{H+} \quad (5)$$

For acid substances So is found by plotting S against $\frac{1}{[H+]}$ and extrapolating the straight line to $\frac{1}{[H+]} = 0$. Using this method pKa values were calculated to be 2.85 (± 0.1168) and 8.27 (± 0.2275).

b) Spectroscopic Method - Table 5 shows the data obtained from the spectrum analysis of ABPP in different pH media and the pKa values calculated from two analytical wavelengths.

Partition Coefficient

Fig 5 shows the partition coefficient/pH profile of ABPP at 25°c. The shape is approximately the inverse of the solubility/pH curve, as expected. The unionised, insoluble, non polar form of ABPP has a higher partition coefficient indicating a greater affinity for Octanol whereas the ionised forms at either end of the pH spectrum favoured aqueous phases.

Stability

The developed HPLC method was used for monitoring both ABPP and ABPP products. The stability studies were mainly concerned with autoclave sterilisation and shelf life stability under conditions of normal stress. The drug was found to be stable to autoclave sterilisation (15 lbs^{-2} for 30 min) and for more than 8 months (experimental period) under normal shelf conditions (Fig 6). This enabled the solutions to be prepared in advance without any risk of a decrease in the labelled potency.

Formulation

In view of the above solubility data and stability and sterilisation results a prototype formulation containing 20 mg/ml of ABPP in a 0.2M Na_2CO_3 solution containing 10% DMA was chosen and subsequent pharmacokinetic work in animals was carried out using this formulation.

Precipitation *in vivo* is a function of the drug concentration of the administered solution, the rate of administration and the effect of other constituents of the blood, e.g. proteins which may retard or prevent

TABLE 5

Determination of pKa Values of ABPP by Spectrophotometric Method

pH	Absorbance (λ 270)	pKa	Absorbance (λ 310)	pKa
11.11	0.110 (d_i)	-	0.163 (d_i)	-
10.73	0.113	8.56	0.165	8.97
9.97	0.112	8.62	0.165	8.21
9.36	0.113	8.20	0.170	8.17
9.15	0.116	8.32	0.177	8.29
8.59	0.149	9.28	0.254	9.15
7.41	0.153	8.45	0.262	8.18
6.52	0.156	8.18	0.273	7.78
5.96	0.158	8.60	0.277	7.74
5.01	0.157 (d_i)		0.279 (d_i)	
		pKa Average = 8.53 ± 0.272		pKa Average =8.31± 0.474
4.66	0.196 (d_m)	-	0.285 (d_m)	-
4.26	0.190	3.37	0.283	2.81
3.06	0.211	2.82	0.269	2.63
2.15	0.237 (d_i)		0.226 (d_i)	
	pKa Average = 3.18		pKa Average = 2.73	

d_i = absorbance of ionised drug

d_m = absorbance of unionised drug

The protolytic constants thus determined are in agreement with the calculated values of 8.27 and 2.85 by using solubility data.

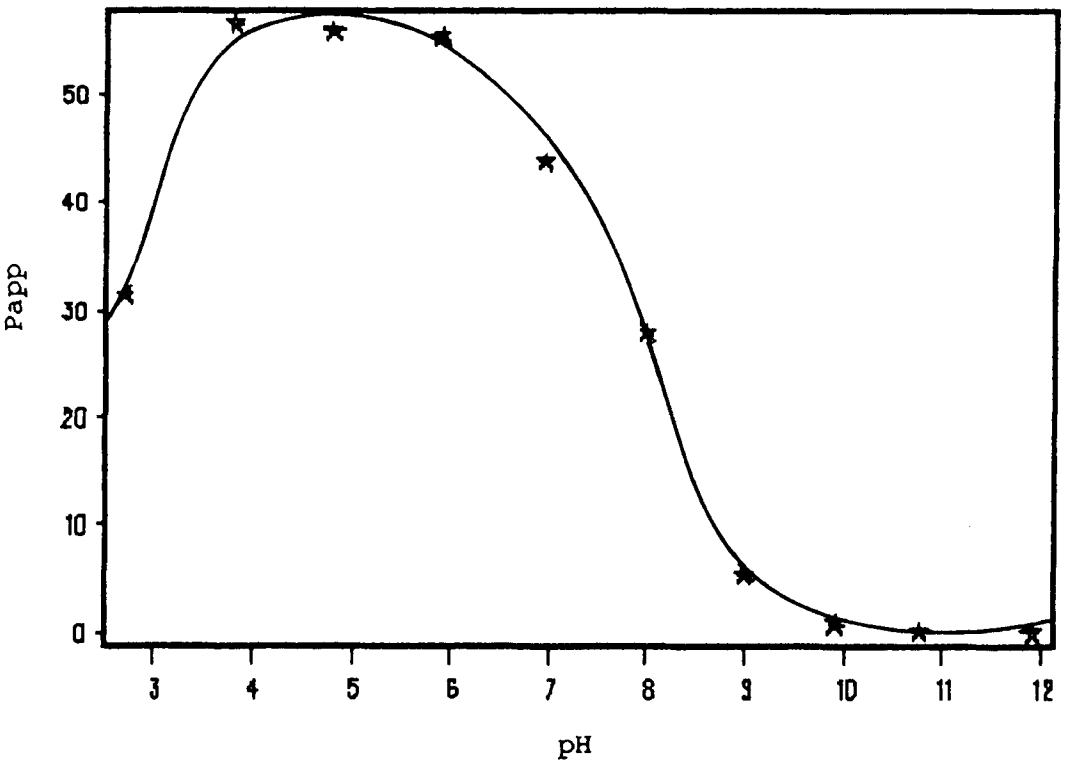

FIGURE 5

APPARENT PARTITION COEFFICIENT OF ABPP as a FUNCTION of pH

precipitation. Therefore, prior to animal studies in vitro tests were carried out to show that no precipitation would occur when the product was mixed with plasma, plasma substitutes and intravenous infusion solutions. In each case no spontaneous or late precipitation were detected. Although the osmolarity of this formulation was high (1930 mosm/kg) no distress was apparent in the rabbits when injected very slowly.

Pharmacokinetic Studies

Intravenous administration of ABPP to rabbits gave high, short lived peaks. Fig 7 is a typical curve showing in the serum ln level curves for 25mg/kg dose level.

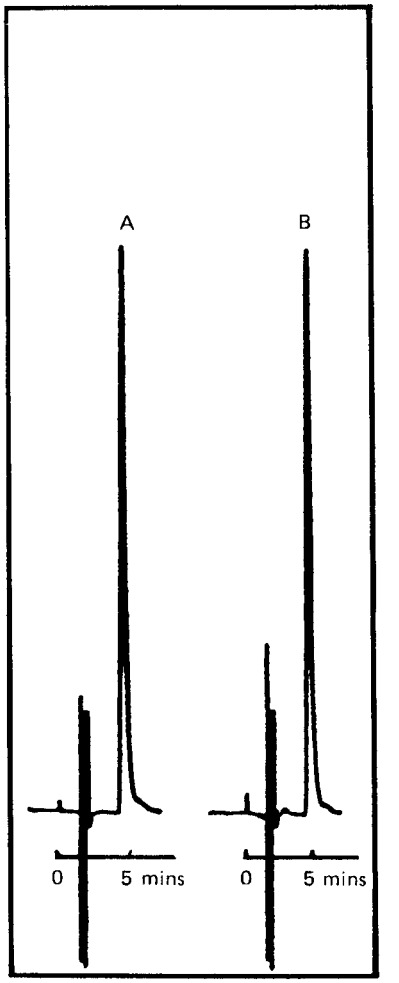

FIGURE 6

CHROMATOGRAM SHOWING STABILITY OF ABPP

A = ABPP at Time O

B = ABPP After 8 Months at Room Temperature in 10% DMA in 0.2N Na_2CO_3

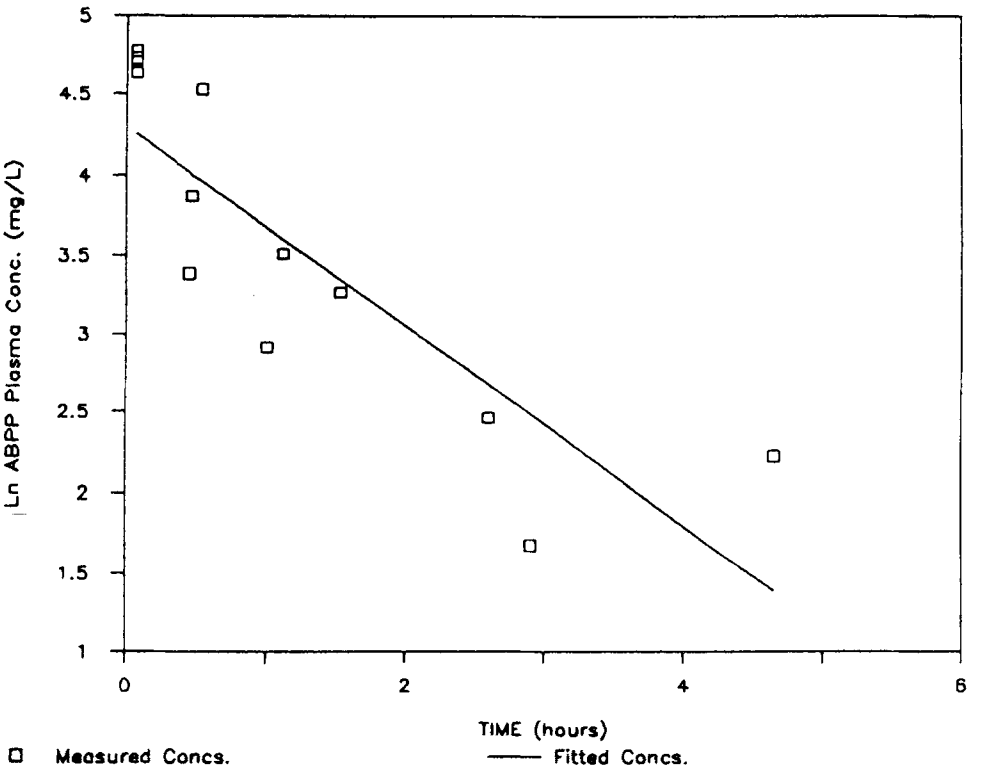

FIGURE 7

ABPP RABBIT PHARMACOKINETICS (AVERAGED)

Dose 25 mg/kg IV bolus.

The apparent elimination half life of ABPP (0.8h) appeared not to be dose related with a straight line relationship between (a) area under the curve and dose and also between (b) C_{pmax} and dose obtained (r_a = 0.9967, r_b = 0.996).

Detailed pharmacokinetic studies related to this formulation will be published in the near future.

CONCLUSIONS

In conclusion, an injectable formulation was prepared providing a dose of 20 mg/ml ABPP using a solvent comprising 10% DMA in 0.2M Na_2CO_3, at a pH 10.4.

The pKa and partition coefficient values add to the existing data concerning ABPP which will be very useful when considering further formulation, administration and mode of action studies.

However, the formulation prepared allows very much needed animal experiments to be carried out using the i.v. route and lays a foundation for further developments.

ACKNOWLEDGEMENTS

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