




*A novel 5-fluorouracil
prodrug using
hydroxyethyl starch as
a macromolecular
carrier for sustained
release*

ABSTRACT

- The objective of this study was to develop a sustained-release drug delivery system for 5-fluorouracil (5- FU) to improve its short half-life.
- 5-Fluorouracil-1-acetic acid (FUAC) was prepared and then conjugated to hydroxyethyl starch (HES) through ester bonds.
- The conjugates were relatively stable in acidic buffer solution at pH 5.8 and slowly released FUAC but became more sensitive to hydrolysis with an increase in the pH and temperature.

Introduction

- ❖ 5-Fluorouracil (5-FU), a low molecular weight drug, is widely used in the treatment of various kinds of cancer, especially solid tumors .
- ❖ Un suitable properties of 5-FU:
 1. There is little selectivity in its pharmacological action  systemic toxicity .
 2. The short in vivo half-life  a sustained therapeutic effect cannot be achieved.
- ❖ To improve an unsuitable property like this, macromolecular prodrugs have been developed.

- ❖ The low molecular weight drugs distribute throughout the body.
- ❖ also, the disposition of macromolecules in vivo depends on both
 1. their physicochemical and biological properties,
 2. and anatomical and physiological characteristics of the body.
- ❖ When antitumor drugs of low molecular weight are coupled with water soluble carriers of high molecular weight 
 - a) the uptake of the formed conjugates is blocked by the capillary endothelium in most tissues.
 - b) a high permeability to most solid tumors because of neovascularization.

❖ To be suitable to be transported selectively to the tumor., a macromolecular carrier must be:

- a. biodegradable and biocompatible,
- b. lack any antigenic and toxic effects,
- c. without any accumulation in the body,
- d. and contain suitable functional groups to allow chemical conjugation .

❖ Hydroxyethyl starch (HES)  shown to be safe and biocompatible in vivo .

- ❖ In the present study, 5-fluorouracil-1-acetic acid (FUAC) was introduced as a 5-FU derivative which had been shown to be highly effective with a low toxicity in a earlier study.
- ❖ The antitumor activity of FUAC was such that it could inhibit:
 - a) the colony formation of leukemia cells (L1210)
 - b) and the growth of transplanted tumor sarcoma 180 (S180), hepatic carcinoma (HEPA) and ehrlich ascites (EC) tumor
- ❖ even at a low concentration (1.0×10^{-5} g/ml).

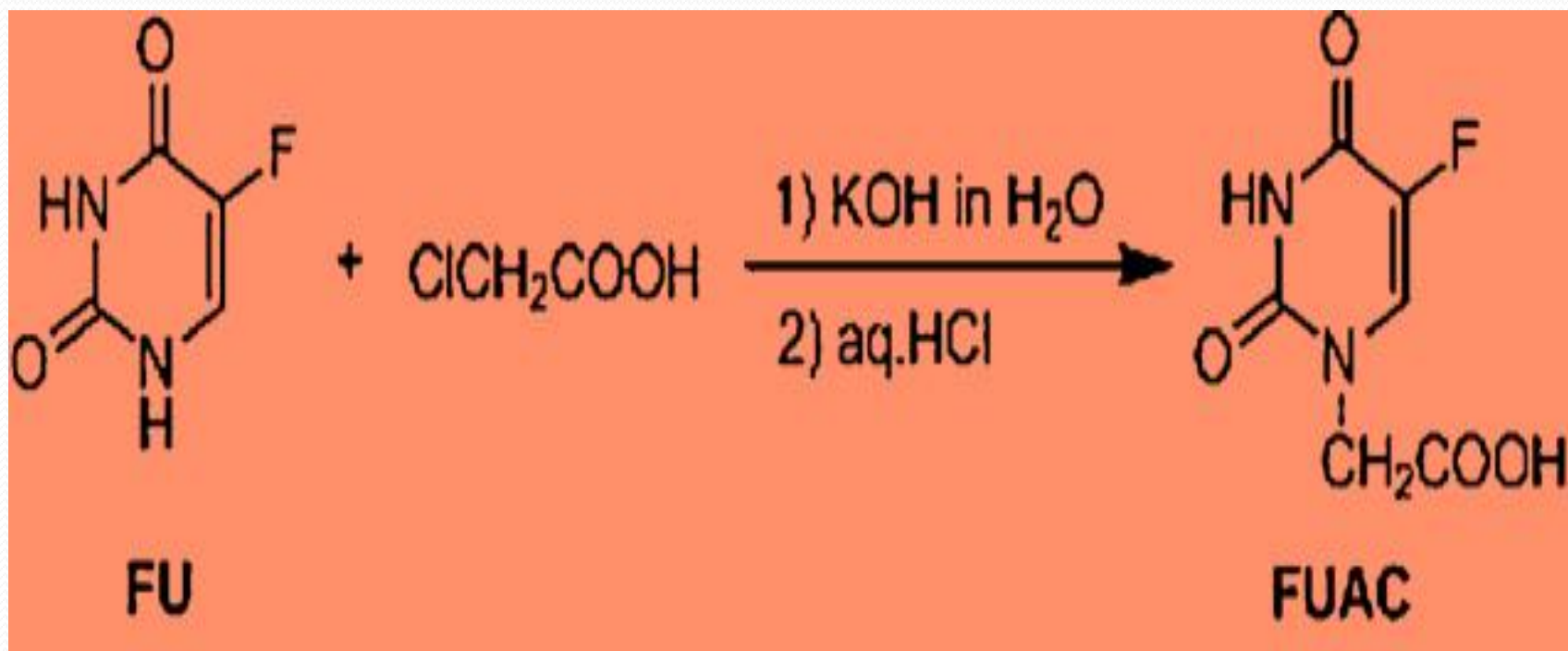
METHODS:

❖ Synthesis of 5-fluorouracil-1-acetic acid (FUAC)

- an improved synthetic approach was developed in which 5-fluorouracil (1.54 g) was dissolved in KOH (2.564 g) aqueous solution (8.0 ml) at 50 °C for 30 min.

- Chloroacetic acid (1.7 g) was then added to the solution slowly over 30 min, and the mixture was stirred at 60 °C. The reaction was monitored by fluorescence thin-layer chromatography.
- After 5 h, the reaction solution was cooled to room temperature, and then hydrochloric acid was added to adjust pH to 5.5, followed by cooling to 4 °C for 2 h.
- Unreacted 5-fluorouracil was removed by filtration. More hydrochloric acid was added to adjust the pH to 2, followed by cooling for 12 h. Then, the formed precipitate was filtered, and washed with distilled water three times.

- After crystallization, needle-like crystals were obtained after drying. The melting point was determined using an X-2 micromelting point apparatus.

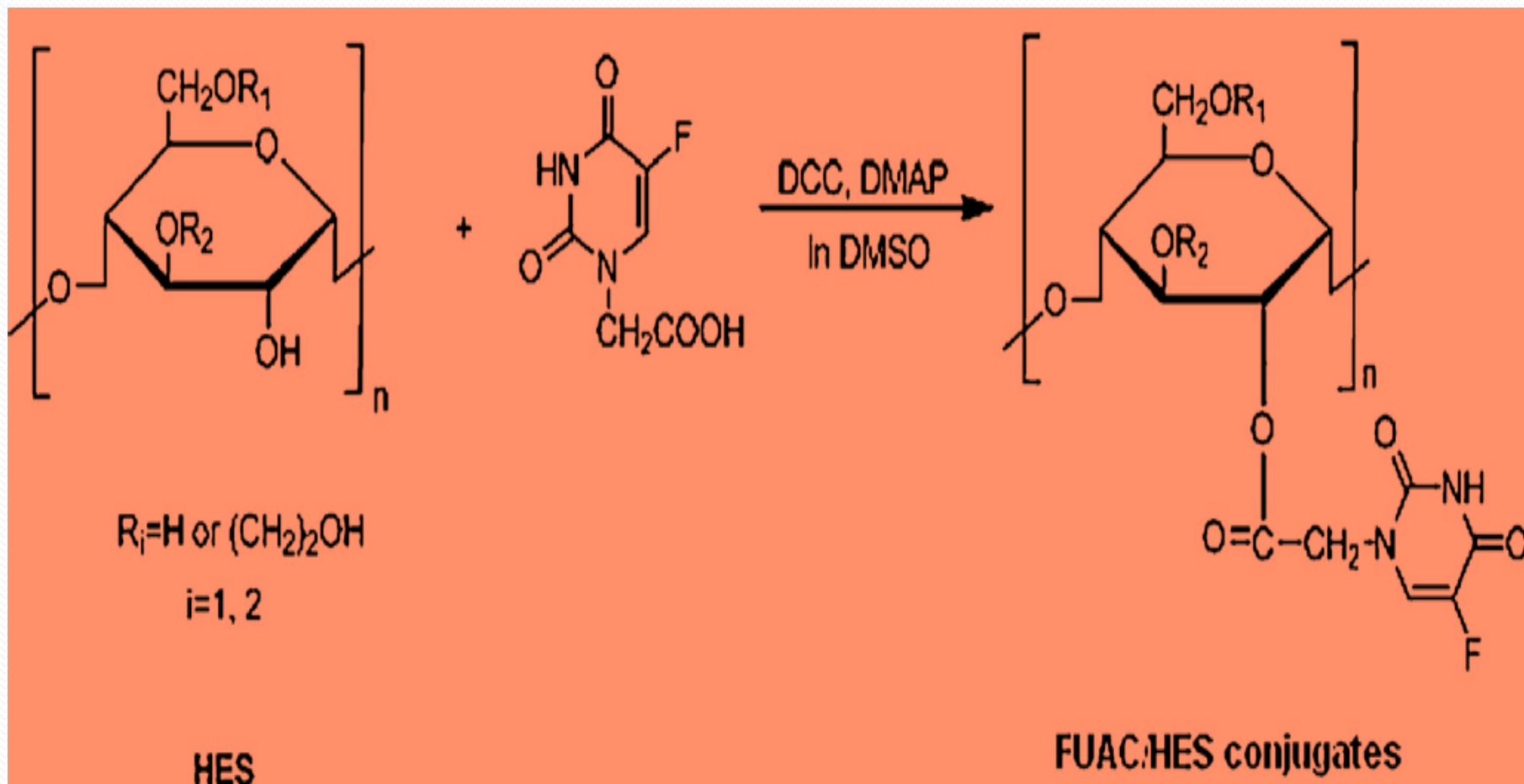


Scheme 1. Synthesis and chemical structure of FUAC.

❖ **Synthesis of 5-fluorouracil-1-acetic acid and hydroxyethyl starch conjugates
(FUAC/HES conjugates)**

- To a solution of HES (1.43 g, in dry DMSO, 20 ml), 5-fluorouracil-1-acetic acid (0.57 g) was added slowly.
- After dissolution, DCC (0.7 g) and DMAP (0.038 g) were added with stirring, and the reaction continued for 24 h at room temperature with protection from light. The generated dicyclohexylurea (DCU) was removed by filtration.
- The conjugates were precipitated after addition of a diethyl ether/dehydrated alcohol (1:3, v/v) mixture (200 ml), and washed with 50 ml diethyl ether. Then, the precipitate was re-dissolved in water, and purified by Sephadex G-50 column chromatography.

- The final conjugates were obtained as a white powder from the eluate after freeze-drying in a Freeze Dryer, and stored in a desiccator.



Scheme 2. Synthesis and chemical structure of FUAC/HES conjugates.

❖ Characterization of the FUAC/HES conjugates and determination of the FUAC content of the conjugates:

- FTIR spectroscopy and NMR spectroscopy were performed to confirm the ester-bond formation.
- The obtained conjugates were characterized using ¹H NMR and IR spectroscopy .
- Thermal analysis was performed by DSC at a heating rate of 10 °C/min with a closed aluminium pan system over the temperature range of 30–300 °C.

- It was found that the conjugates were completely hydrolyzed to release FUAC in strongly alkaline solution.
- Therefore, the drug content was determined by adding 20 ml NaOH 2 mol/L to 10.0 mg conjugate at a temperature of 60 °C with magnetic stirring.
- After 1 h, the reaction solution was cooled to room temperature, and 20 ml HCl (2 mol/L) was used to stop the reaction and neutralize the solution.
- The content of FUAC was calculated by comparison with the FUAC standard curve.

Hydrolysis in phosphate buffered saline

(PBS):

- Conjugates were dissolved in a series of various PBS solutions (0.01 mol/L) at pH 1.2, 2.5, 5.8, 7.4, 8.0, 10.0 to give a final concentration of 0.5 mg/ml.
- The buffer solutions were incubated at 37 °C for 12 h and 0.1 ml portion of each reaction solution was filtrate
- The each filtrate was analyzed by HPLC.
- The retention time of the 5FU, FUAC, and FUAC/HES conjugates was 3.0 min, 3.8 min, and 8.8 min, respectively.
- The same procedure was also carried out at 60 °C in the series of PBS solutions.

Results and discussion

□ Synthesis and characteristic of FUAC/HES conjugates:

- FUAC was prepared with a melting point at 278 °C measured in a micromelting point apparatus .
- FUAC/HES conjugates were synthesized using DMAP as an acyl transfer catalyst, and DCC as a condensing agent, and purified by Sephadex column chromatography in high yields (>95%) without any free FUAC.

- ^1H NMR (DMSO) shown in Fig. 1: 1 (ppm) 3.2–3.75 and 4.45–4.9 (CH–OH. and –CH₂O, HES), 3.35 (N–CH₂–COO–), 5.14 (CH–OCO), 7.94 (C₆–H, FU).
- The peaks from the IR analysis (KBr) were as follows: 3420 (O–H), 2925 (C–H, stretch, aliphatic and alkene) 1700 (C O, ester), 1675 (C O, carboxylic acid), 1609, 1510 (C C), 1370 (C–N), 1250 (C–F).

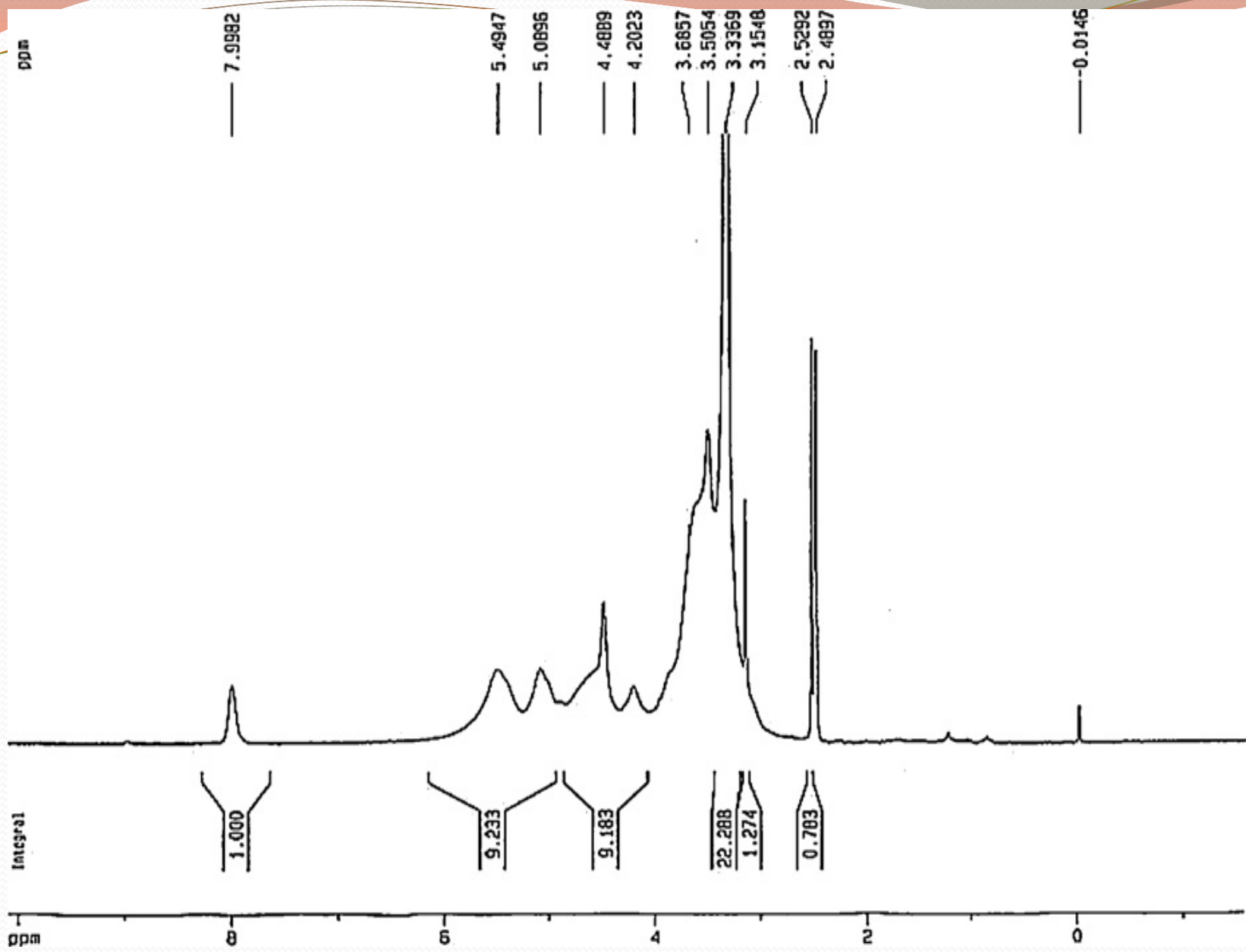


Fig. 1. NMR spectrum of FUAC/HES conjugates.

- As seen in Fig. 2, 5-FU has an exothermic peak at 290 °C. The exothermic peak of FUAC shifted to 281 °C showing that the derivative of 5-FU was generated after carboxymethylation.

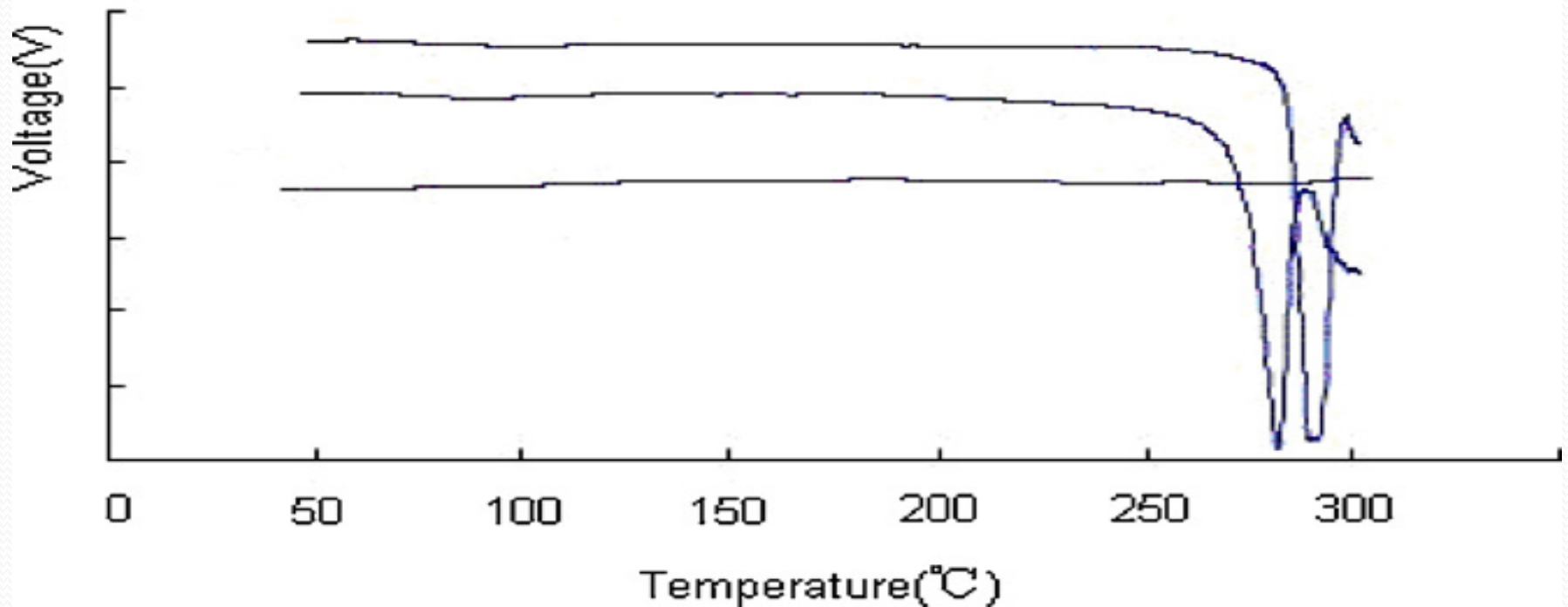


Fig. 2. DSC thermogram of samples, from top to bottom: 5-FU, FUAC, and FUAC/HES conjugates.

- The DSC thermogram of the final FUAC/HES conjugates was smooth and no characteristic peaks of 5-FU or FUAC appeared which suggested that linking the drug molecule to the amorphous carrier reduces the degree of drug crystallization so that there were no exothermic peaks.
- All these results confirmed that FUAC was successfully covalently bound to HES.
- The content of FUAC loaded in the conjugates depends on the hydroxyl groups in HES and the reaction conditions without water.
- After repeated experiments, the content was basically stable at 15% meaning that 15 mg FUAC was loaded in 100 mg conjugate.

□ In vitro stability of FUAC/HES conjugates:

- As 5-FU was not detected in any buffer, FUAC/HES conjugates degraded according to first-order kinetics releasing FUAC as same as the hydrolysis of FUAC and other macromolecular conjugates.
- Stability studies performed in the various media using 12 h incubation showed that no matter what the temperature of the media was, the conjugate was relatively stable in acidic buffer solution at pH 5.8 in which the FUAC cumulative release was less than 3% at 37 °C and 18.0% at 60 °C.

- However, it was more sensitive to hydrolysis when the pH changed from 7.0 to 10.0.
- The observed hydrolysis rate constant (k_{obs}) could be calculated for FUAC using the pseudo-first-order kinetics equation.
- The results shown in Table 1 indicate that a specific base-catalysis was involved in the hydrolysis of the ester conjugate which was usually observed in weak alkaline solution in previous studies.

Table 1:

Kinetic data for hydrolytic degradation of conjugates in PBS at different pH values at 37 °C and 60 °C.

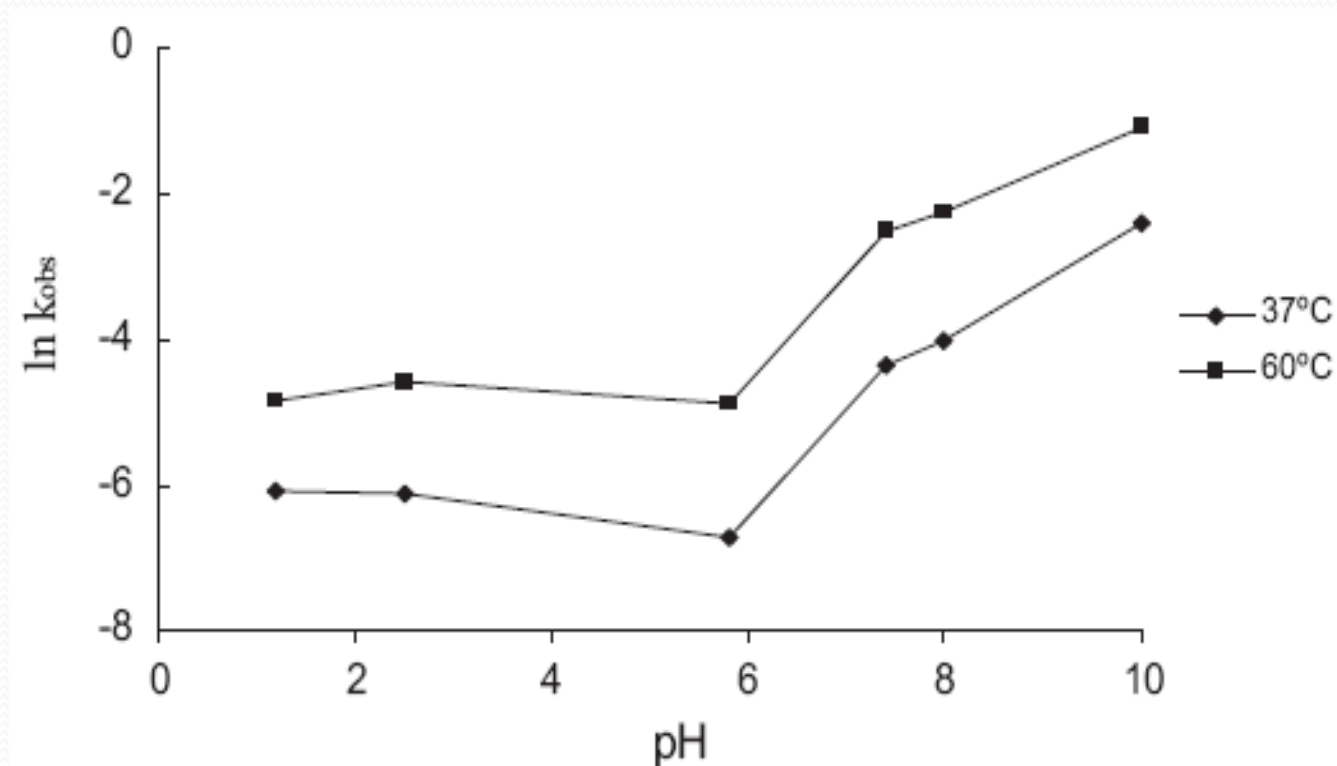
pH value	37 °C		60 °C		Eobs (kJ/mol)
	kobs (1/h)	t1/2 (h)	kobs (1/h)	t1/2 (h)	
1.2	0.0023	301.3	0.0079	87.7	46.85
2.5	0.0022	315.0	0.0101	68.6	57.86
5.8	0.0012	577.5	0.0076	91.2	70.07
7.4	0.0130	53.3	0.0812	8.5	69.54
8	0.0178	38.9	0.1041	6.7	67.05
10	0.09002	7.7	0.3408	2.0	50.54

kobs, the observed hydrolysis rate constant; t1/2, half-life; Eobs, the observed activation energy.

- It is known that the hydroxide ion supplied as a strong nucleophile by the media directly attacks the ester carbonyl leading to transfer of the hydrolysis balance of the ester bond irreversibly increasing the hydrolytic rate constant.
- Another reason may be that the intramolecular hydrogen bonding is affected by the adjacent hydroxy groups which prevents the hydrolytic degradation.
- With an increase in the pH, the hydrogen bond is destroyed and the hydrolysis is accelerated.

- The degree of hydrolysis was also related to the temperature of the medium.
- Hydrolysis was more marked at 60 °C under the same conditions.
- According to the Arrhenius equation, the observed activation energy (E_{obs}) could be calculated from the data at 37 °C and 60 °C.
- The average of the observed activation energy was 60.32 ± 10.08 kJ/mol.

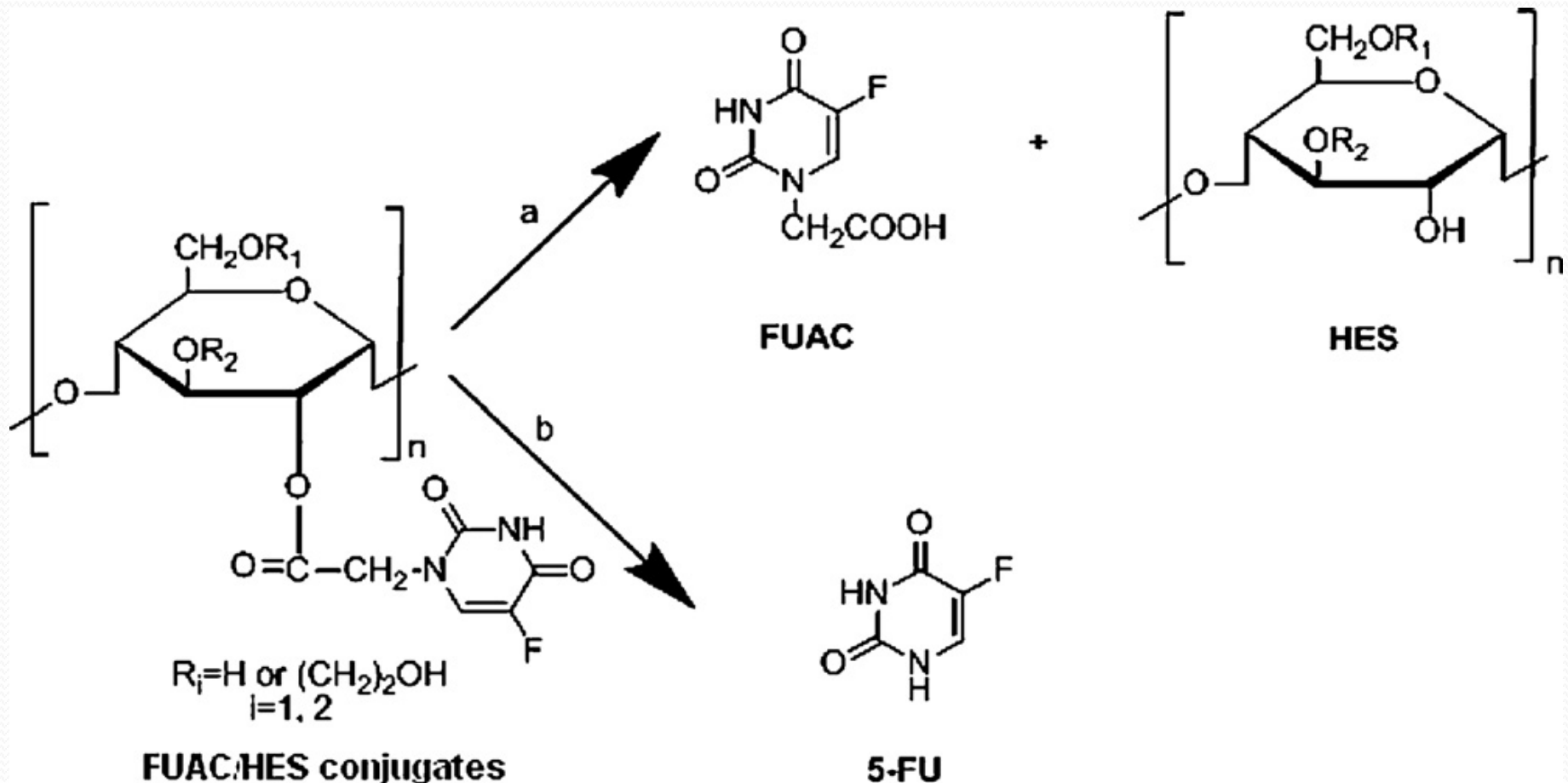
- Fig. 3 clearly shows that the degree of hydrolysis is strongly dependent on the pH and temperature.



k_{obs} ,The observed hydrolysis rate constant

Fig. 3. Plots of rate constant versus pH in PBS with different pH values at 37 °C and 60 °C.

The degradation to 5-FU from the conjugate may be carried out chemically or enzymatically in the liver via the different processes shown in Scheme 2.



Scheme 2. The enzymolysis in rat liver homogenate of FUAC/HES conjugates.

- The probable mechanism involves the nucleophilic attack on the carbon connected to the nitrogen or possible hydroxylation of the carbon leading to free 5-FU, although the exact mechanism of the release of 5-FU from FUAC/HES conjugate remains unknown.
- A 5- fluorouracil-1-acetic acid human serum albumin conjugate was synthesized which was also degraded to 5-FU in liver homogenate, but no 5-FU was found in rat liver homogenate when FUAC was incubated for 24 h.
- We believe that it is easier to release 5-FU when coupled to a macromolecule compared with FUAC.

Conclusions:

- The low molecular weight anticancer drug FUAC was initially successfully linked to hydroxyethyl starch through an ester bond.
- FUAC, not 5-FU, was released from the conjugate in vivo.
- The 5-FU prodrug of HES as a carrier for injection was potential to make up for the deficiency of the tedious injection of 5-FU with high doses to reach effective therapy but always accompanied with toxicity.
- The pharmacokinetic study indicated that the conjugates exhibit clear sustained-release of FUAC and have potential clinical applications for the treatment of tumors.