the piperazine-2,5-dione was separated from the ordinary components of human urine on a C_{18} column using phosphate buffer-methanol (pH 3·5) as an eluent. Subsequently, the effluent was led to a post-column reaction with mercurous chloride, and then detected at 305 nm. At a concentration of 1·0 μg ml^{-1} of the piperazine-2,5-dione in human urine, the precision was of the order of 2·0% (n = 5). The proposed method permits detection of 0·1 μg ml^{-1} in human urine.

Under the HPLC and post-column reaction conditions mentioned above, a 20 μl portion of the 2–3 h urine after oral administration of ampicillin was injected into the HPLC (Fig. 1b). The retention time of peak 1 substance agreed with the authentic sample of the piperazine-2,5-dione revealing that it is also excreted in the urine of humans dosed with ampicillin. The maximum excretion rate of the piperazine-2,5-dione was 1·35 mg h^{-1} as parent penicillin equivalent, which was attained at 3 h after administration and its cumulative amount was 3·08 mg until 8 h as parent penicillin equivalent, which accounted for 0·62% of the dose. The proposed HPLC method will be useful for the assay of the piperazine-2,5-dione in serum with slight modifications.

REFERENCES

Bird, A. E., Cutmore, E. A., Jennings, K. R., Marshall, A. C. (1983) J. Pharm. Pharmacol. 35: 138–143

Bundgaard, H., Larsen, C. (1979) Int. J. Pharmaceutics 3: 1–11

Everett, J. R., Jennings, K. R., Woodnutt, J., Buckingham, M. J. (1984) J. Chem. Soc., Chem. Comm. 894–895

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Uridine-induced hyperthermia in the rabbit

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Uridine injection in 0·6% saline elevated rabbit temperatures (mean = 0·9 °C) in the USP XX pyrogen test. Hyperthermia was delayed in onset and peaking 3–4 h post injection, but the injection was negative in the limulus amoebocyte lysate (LAL) assay. Uridine from five lots of different sources exceeded USP XX limits in the rabbit pyrogen test and proved negative in the LAL assay. Because the dose of uridine was high, several procedures were used to determine if an impurity was the cause of temperature elevation. Uridine remained pyrogenic in spite of ultrafiltration (10 000 nominal mol. wt), recrystallization and preparative scale HPLC. Sterile filtration and autoclaving also did not affect the response. Hyperthermia, therefore, appears to be an inherent property of uridine. Uridine was also found to release endogenous pyrogen in-vitro from human mononuclear cells. Uridine has been reported to induce fever in man, thus the USP rabbit pyrogen test predicted for the clinical response.

Generally, good agreement exists between the two United States Pharmacopeial pyrogen methods, the in-vivo rabbit test and the in-vitro limulus amoebocyte lysate procedure (LAL). Discrepancies appear usually as positive limulus and negative rabbit tests due to the greater sensitivity (about ten-fold) of the LAL method. However, several compounds (e.g. bleomycin, muramyl dipeptide) induce fever in rabbits but are negative or weakly positive in the limulus test (Dinarello & Wolff 1982).

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Martin et al (1982) reported that high doses of the endogenous nucleoside, uridine, reversed toxicity induced by 5-fluorouracil in mice without affecting antitumour activity. Those workers subsequently designed a clinical protocol that would utilize a continuous intravenous (i.v.) infusion of uridine. In the process of preparing and evaluating a sterile large volume parenteral dosage form for use in that clinical trial, we found that uridine produced hyperthermia in rabbits at doses projected to be reached clinically (>300 mg kg^{-1}). However, the injection solution was negative by the LAL method. This report describes an investigation of the hyperthermia induced by uridine in rabbits as well as an evaluation of impurities as a causal factor.

Methods

Rabbit pyrogen tests were carried out essentially following the USP XX procedure with three rabbits and hourly measurements of colonic temperature. Some of the pyrogen tests were conducted by South Mountain Laboratories, S. Orange NJ. A test was considered positive if the peak temperature increase in one of three rabbits was ≥0·6 °C or if the sum of the increase was >1·4 °C during the 3 h observation period required by the USP. In some experiments the observation period was extended up to 7 h post injection. US standard endotoxin (EC-5), 30 EU kg^{-1}, and 0·9% sodium

chloride injection USP, 10 ml kg^{-1} were used, respectively, as positive and negative controls.

Samples of uridine were obtained from the following sources: Aldrich Chemical Company, Milwaukee, WI; Calbiochem, La Jolla, CA; Leon Industries, St. Charles, MO; Sigma Chemical Company, St. Louis, MO; and Yamasa Shoyu, Tokyo, Japan. Most experiments were run on a single lot (I) of uridine. In view of the positive pyrogen response obtained with repeated testing of this lot at 300 mg kg^{-1} in several laboratories, uridine was obtained commercially from four other sources (lots II–V). Usually 3% solutions of uridine were prepared in 0·6% sodium chloride and injected at 10 ml kg^{-1}. However, 6% uridine solutions were used to deliver 450 or 600 mg kg^{-1} doses at 7·5 or 10 ml kg^{-1}. Some solutions were subjected to sterilizing filtration (0·22 μm) and autoclaving (121 °C for 15 min) to simulate processing conditions.

Uridine solutions were tested for endotoxin using the limulus amoebocyte lysate assay procedure (Cooper 1975). Equal volumes (0·1 ml) of drug solution and lysate were incubated at 37 °C for 60 min in 8 × 75 mm test tubes. A positive result was a firm gel that could withstand 180° inversion of the tube. No inhibition of gel formation was detected at 30 mg ml^{-1} of uridine, but some interference was noted at ≥100 mg ml^{-1} uridine. LAL test sensitivity was increased by using an ultrafiltration method (Takahashi et al 1983) to concentrate any residual endotoxin and separate this material from uridine. A 10 ml portion of uridine solution, 200 mg ml^{-1}, was filtered through an Amicon PM10 membrane (10 000 nominal molecular weight) and followed by filtration of 15 ml of sterile water to concentrate any endotoxin from the 2 g sample into 1 ml. LAL tests were then run on the 1 ml retentate.

Purification procedures. Conventional chemical analysis of the uridine sample by elemental analysis, ultraviolet spectroscopy, thin layer and high pressure liquid chromatography (HPLC) indicated that lot I was of high chemical quality (>99·5%) and exceeded the supplier's claim. However, pyrogens are very potent—pyrogenic doses of US reference endotoxin (Rudbach et al 1976) and muramyl dipeptide-MDP (Dinarello et al 1978) are, respectively, about 1 ng and 100 μg. Three procedures were used in an attempt to separate uridine from any pyrogenic contaminants: ultrafiltration, preparative scale HPLC, and recrystallization. The samples were tested by the LAL method to rule out inadvertent introduction of endotoxin during processing.

Ultrafiltration. A 3% uridine solution was prepared in sterile water for injections, filtered through a 0·22 μm membrane and then ultrafiltered under nitrogen pressure through a 10 000 nominal molecular weight membrane (Amicon PM 10). The ultrafiltrate was freeze-dried, dissolved in 0·6% sodium chloride injection to yield a 3% solution and then evaluated in both the LAL and USP XX rabbit pyrogen tests.

Preparative HPLC. Lot I was converted to the corresponding triacetate derivative by treatment with pyridine and acetic anhydride at room temperature (20–23 °C) over 24 h. The triacetate was recrystallized from chloroform–ether (1 : 1). NMR and infrared spectra were consistent with the structure. The triacetate derivative was subjected to preparative scale HPLC using a silica gel column (Prep Pak-500) and an eluent of ethyl acetate–methylene chloride (1 : 1). Fractions corresponding to ascending and descending sections of the peak were collected and discarded. The middle fraction was evaporated to dryness and recrystallized from chloroform–ether at 85% yield. The triacetate derivative recovered from HPLC was then hydrolysed to uridine by treatment with methanolic ammonia in an ice bath. The solid was twice recrystallized from a mixture of methanol and ethanol to yield 5·5 g of uridine that was identical to the original uridine sample as determined by NMR and infrared spectra, melting point and mixed melting point. Another sample of lot I was treated with charcoal and twice recrystallized from ethanol.

Release of leucocytic pyrogen. Leucocytic pyrogen is released from mononuclear phagocytes upon appropriate stimulation and is thought to mediate the febrile reaction elicited by a variety of pyrogenic substances (Dinarello 1981). The potential for uridine to release leucocytic pyrogen was evaluated using the procedure of Ackerman et al (1984). Cultures of human mononuclear cells were exposed to uridine, 3 and 30 mg ml^{-1}, for 16 h in-vitro at 37 °C. Supernatant (1 ml) was then injected into rabbits and the temperature was recorded according to USP XX procedures. Since rabbits respond to human leucocytic pyrogen with a febrile response, fever indicates release of leucocytic pyrogen by the mononuclear cells.

In an attempt to induce tolerance, six rabbits were injected i.v. daily for 5 days at a uridine dose of 300 mg kg^{-1}. The pyrogen test was run on days 1, 3 and 5.

Results

Pyrogen test results for lot I at 300 mg kg^{-1} are listed in Table 1. Uridine was reproducibly pyrogenic according to USP XX criteria. Interestingly, the peak temperature increase was observed at the third hour after injection, and essentially no fever was observed at the first hour.

Table 1. The effect of uridine (lot I 300 mg kg^{-1}) on rectal temperature in the rabbit (USP XX pyrogen test)*.

	Time (h)		
Test	1	2	3
A	+0·1*	+0·3	+0·7
B	−0·2	+0·4	+0·8
C	−0·2	+0·5	+0·9

* Average change in °C, n = 3.

Usually a slight decrease (about 0·2 °C) was noted from pre-injection values. In several tests the observation time was extended beyond the 3 h required by the USP. Temperatures were recorded hourly, and the maximum increase was observed at 3 and 4 h with a return to pretreatment values noted 6 h post injection (Fig. 1). Endotoxin yielded a biphasic temperature curve typical for high doses of this substance (Greisman & Hornick 1969).

After demonstrating that the large batch of uridine (lot I) produced fever in rabbits, four other lots were obtained from separate sources. All five lots were pyrogenic by USP criteria at 300 mg kg^{-1} (Table 2). The average increase per rabbit was 0·9 °C. The magnitude of the hyperthermia was similar between the five lots. At least ten pyrogen tests have been carried out at a 300 mg kg^{-1} dose. In all cases the results exceeded USP XX limits for a three-rabbit test. Several other doses were examined. The average temperature increase per rabbit at 200, 300, 450 and 600 mg kg^{-1} was, respectively, 0·1, 0·8, 1·5 and 0·8 °C. The response was within USP limits only at 200 mg kg^{-1}.

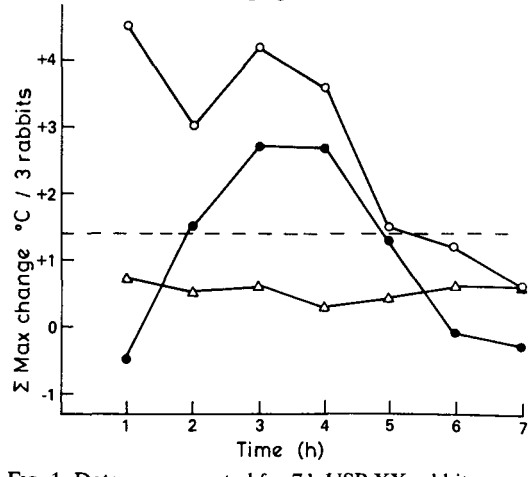

FIG. 1. Data are presented for 7 h USP XX rabbit pyrogen test for uridine, 300 mg kg^{-1} (●); United States reference endotoxin (EC 5), 30 EU kg^{-1} (○) and 0·9% sodium chloride injection, USP, 10 ml kg^{-1} (△), The USP XX limit for a three rabbit test is indicated by the dotted line (sum of maximum temperature increases ≤1·4 °C).

Table 2. USP XX pyrogen test of uridine 300 mg kg^{-1} [a].

Lot	Hours 1	2	3	Total[b] (°C)
I	0	+0·42	+0·77	2·4
II	−0·73	+0·1	+0·83	2·5
III	+0·17	+0·6	+0·73	2·4
IV	+0·05	+0·65	+0·77	2·4
V	+0·13	+0·85	+1·13	3·4

[a] Each value is the mean temperature change of three rabbits except for lot I where n = 12. All lots tested were negative at 30 mg ml^{-1} in the limulus test.

[b] Sum of the maximum temperature increase per three rabbit test.

Table 3. Effect of treatment of lot I on uridine-induced hyperthermia in rabbits[1].

Treatment	Max increase °C/Rabbit I	II	III	Total
None[2]	0·8	0·7	0·9	2·4
Sterile formulation[3]	0·7	0·7	0·8	2·2
Ultrafiltration[4]	1·6	1·2	0·4	3·2
Recrystallization[5]	0·9	0·7	0·6	2·2
Preparative scale HPLC[6]	0·7	0·7	0·6	2·0

[1] Tests were conducted using the USP XX method at 300 mg kg^{-1}.

[2] Lot I was dissolved without further purification in 0·6% sodium chloride solution to yield a 3% solution.

[3] Lot I was dissolved in 0·6% sodium chloride solution, filtered through a 0·22 μm membrane and autoclaved for 15 min at 121 °C.

[4] Lot I was successively filtered through a 0·22 μm membrane and an Amicon 10 000 mol. wt filter, freeze dried and then reconstituted to 3% in 0·6% sodium chloride solution.

[5] Lot I was treated with charcoal and twice recrystallized from ethanol, dried and dissolved in 0·6% sodium chloride.

[6] Uridine was derivatized to the triacetate, subjected to preparative scale HPLC using a silica gel column and eluted with ethyl acetate–chloroform 1 : 1. The fraction corresponding to the triacetate was isolated, hydrolysed back to uridine and twice recrystallized. Uridine was then dissolved at 3% in 0·6% sodium chloride solution. See Methods for further details.

Both the injection solution (30 mg ml^{-1} uridine) and the retained concentrate from ultrafiltration (equivalent to 2 g ml^{-1} uridine) were negative in the LAL test. Drug interference was not observed with either method.

Lot I was subjected to several procedures to find if an impurity caused hyperthermia. In spite of: (1) pharmaceutical processing of a sterile solution, (2) ultrafiltration, (3) recrystallization and (4) preparatory scale HPLC, lot I remained pyrogenic (Table 3). Limulus tests at 30 mg ml^{-1} of uridine were negative, indicating the samples were not contaminated during handling.

Uridine-induced release of leucocytic pyrogen from human mononuclear cells was studied. Intravenous administration of the supernatant from the 30 mg ml^{-1} uridine sample stimulated release of leucocytic pyrogen as evidenced by a mean febrile response of 0·8 °C (± 0·2 °C, s.d.) in the rabbits, but the control (0·3 ± 0·1 °C ± s.d.) and 3 mg ml^{-1} uridine sample (0·1 ± 0·1 °C, s.d.) were negative. Simultaneous assays using the LAL technique showed that the endotoxin content was inadequate to elicit leucocytic pyrogen release (Ackerman et al 1984). The uridine dose itself (about 15 mg kg^{-1}) was insufficient to induce a positive response in the rabbit.

Tolerance to uridine fever was at 300 mg kg^{-1} uridine i.v. daily for five days. Average temperature elevation observed post drug administration exceeded USP limits on days 1, 3 and 5 (1·38 ± 0·22, 0·88 ± 0·22, 0·87 ± 0·22 °C ± s.e. respectively) and indicated that tolerance to uridine-hyperthermia does not readily occur in the rabbit.

Discussion

Hyperthermia was repeatedly observed after intravenous injection of uridine, 300 mg kg^{-1}, from five separate sources; no other adverse effects were observed. In view of the high doses required to induce this effect, emphasis was placed on evaluation for trace impurities.

The most obvious pyrogenic contaminant in pharmaceuticals is endotoxin, however it does not appear to play a role in uridine-hyperthermia. The 3% uridine solution was negative by LAL tests, but the pyrogen dose was high (300 mg kg^{-1}) and interference was noted at ≥10% uridine. Although the LAL test is recognized as being about ten-fold more sensitive than the USP pyrogen test (Wachtel & Tsuji 1977), Cooper (1975) noted that this enhanced sensitivity applied only to small volume parenterals. There was little difference for pharmaceuticals administered in a large volume (≥500 ml). Since uridine was intended for infusion use, this drug was tested at high concentration (equal to 2 g ml^{-1}) using the ultrafiltration method of Takahashi et al (1983) to circumvent the uridine inhibition of the LAL test. The sensitivity was increased by about sixty-fold and no endotoxin was detected. The time course of uridine-hyperthermia in the rabbit also differs from endotoxin (Fig. 1) and 10 000 mol. wt ultrafiltration had no effect on the pyrogen tests results. Ultrafiltration has been used to separate endotoxin from pharmaceuticals (Cradock et al 1978). The relationship between uridine and hyperthermia more closely resembles the sequence following i.v. injection of bleomycin (Dinarello et al 1973) or MDP (Dinarello et al 1978; Parant et al 1980). In those instances fever is delayed in onset, monophasic in shape and peaks at 3–4 h post injection.

Other purification procedures were also evaluated. In spite of a multistep preparative HPLC treatment, uridine produced hyperthermia in the rabbit. Charcoal treatment and double recrystallization from ethanol also had little effect. Hyperthermia therefore appears to be an inherent property of uridine.

The hyperthermia appears to resemble the characteristics exhibited by bleomycin, muramyl dipeptide and analogues of this immune stimulant, i.e. positive in the rabbit pyrogen test, delayed monophasic fevers, and negative results in the limulus test. Despite differences in kinetics of the fever, the pyrogenic behaviour of bleomycin and MDP, like endotoxin and most other pyrogens, has been attributed to drug-induced release of endogenous pyrogen from leucocytes (Dinarello & Wolff 1982). At a concentration of 30 mg ml^{-1}, uridine behaves similarly, establishing that the mechanism for fever induction is comparable to that of other pyrogens. Since human leucocytes were used in this determination, the data also suggest that uridine may be pyrogenic in man. In fact, von Groeningen et al (1984) recently administered uridine clinically. Fever to 39 °C was noted during continuous infusions of 1 and 2·5 g m^{-2} h^{-1}. The sample identified as lot IV in Table 2 was supplied by these workers and found by us to be positive in the USP pyrogen test.

Use of the LAL method to test pharmaceuticals for pyrogens is increasing. However, the results of this study plus those on MDP and bleomycin (Dinarello et al 1973, 1978) reconfirm the need to continue testing in the rabbit model.

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REFERENCES

Ackerman, S. K., Hochstein, H. D., Zoon, K., Browne, W., Rivera, E., Elisberg, B. (1984) J. Leukocyte Biol. 36: 1727–1737

Cooper, J. F. (1975) Bull. Parenteral Drug Assoc. 29: 122–130

Cradock, J. C., Guder, L. A., Francis, D. L., Morgan, S. L. (1978) J. Pharm. Pharmacol. 30: 198–199

Dinarello, C. A. (1981) Lymphokines 7: 23–74

Dinarello, C. A., Wolff, S. M. (1982) in: Born, G. V. R., Fara, A. Herken, H., Welch, A. D. (eds) Handbook of Experimental Pharmacology, Vol. 60, pp 73–112

Dinarello, C. A., Ward, S. M., Wolff, S. M. (1973) Cancer Chemother. Rep. 57: 393–398

Dinarello, C. A., Elin, R. J., Chendid, L., Wolff, S. M. (1978) J. Inf. Dis. 138: 760–767

Greisman, S. E., Hornick, R. B. (1969) Proc. Soc. Exp. Biol. Med. 131: 1154–1158

Martin, D. S., Stolfi, R. S., Sawyer, R. C., Spiegelman, S., Young, C. W. (1982) Cancer Res. 42: 3964–3970

Parant, A., Riveau, G., Parant, F., Dinarello, C. A., Wolff, S. M., Chedid, L. (1980) J. Inf. Dis. 142: 708–715

Rudbach, J. A., Akiya, F. I., Elin, R. J., Hochstein, H. D., Luoma, M. K., Miller, E. C. B., Milner, K. C., Thomas, K. R. (1976) J. Clin. Microbiol. 3: 21–25

Takahashi, S., Yano, S., Nagaoka, Y., Kawamura, K., Minami, S. (1983) J. Pharm. Sci. 72: 739–742

von Groeningen, C., Leyva, A., Peters, G., Kraal, I., Pinedo, H. (1984) Proc. Am. Assoc. Cancer Res. 25: 169

Wachtel, R. E., Tsuji, K. (1977) Appl. Environ. Microbiol. 33: 1265–1269