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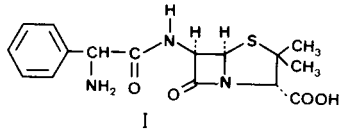
A sensitive high performance liquid chromatographic assay of a new metabolite of ampicillin in man

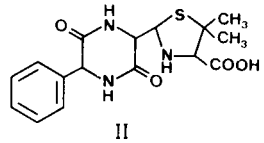
JUN HAGINAKA*, JUNKO WAKAI *Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 4–16 Edagawa-cho, Nishinomiya 663, Japan*

A sensitive, high-performance liquid chromatographic method has been developed for the determination of piperazine-2,5-dione, a new metabolite of ampicillin, in human urine. Piperazine-2,5-dione was separated from human urine on a C18-column using phosphate buffer–methanol (pH 3·5) as eluent. Subsequently, the effluent underwent a postcolumn reaction with mercurous chloride and was then detected at 305 nm. It was found that piperazine-2,5-dione was also excreted in the urine of a volunteer dosed with ampicillin.

It is well known that ampicillin (I) is metabolized to the corresponding 5*R*, 6*R*-penicilloic acid and the 5*S*,6*R*-epimer in man (Bird et al 1983). In a recent investigation of the metabolism of ampicillin by means of spin-echo ^1H nuclear magnetic resonance spectroscopy, a piperazine-2,5-dione (II) was demonstrated as a new metabolite of ampicillin in rat urine (Everett et al 1984).



I



II

This communication reports that the piperazine-2,5-dione is also exreted in human urine after oral administration of ampicillin, and describes a sensitive high-performance liquid chromatographic (HPLC) assay method for its detection in human urine.

Materials and methods

Ampicillin was kindly donated from Meiji Seika Kaisha. The piperazine-2,5-dione was isolated according to the procedures described by Bundgaard & Larsen (1979).

Reversed-phase HPLC was carried out using an LC-5A (Shimadzu) pump, a 15 cm × 4·6 mm i.d. stainless steel column packed with Develosil ODS-5 (5 μm, Nomura Chemicals) and a variable wavelength detector (UVIDEC-100-V, Jasco). The eluent used was 7 mM sodium dihydrogen phosphate plus 3 mM phosphoric acid–methanol (1·5 : 1, v/v) (pH 3·5), whose flow rate was maintained at 0·8 ml min^{-1}. For the post-column reaction, 5×10^{-4} M mercurous chloride was delivered at a flow rate of 0·2 ml min^{-1}. Teflon tubing (0·5 mm i.d. × 1 m) was used as a reactor and detection was at 305 nm. All separations and postcolumn reactions were carried out at ambient temperature.

Ampicillin (500 mg as potency) was orally administered to a healthy volunteer fasted for 12 h. Urine samples were collected at 0, 1, 2, 3, 4, 5, 6, and 8 h and filtered using 0·45 μm acrylate copolymer membranes, and a 20 μl portion of the filtrate was injected into the chromatograph under the conditions described above.

Results and discussion

Bundgaard & Larsen (1979) reported that reaction of the piperazine-2,5-dione with mercurous chloride in solution at pH 7·0 gave the corresponding penamaldate (which has strong ultraviolet absorption at 305 nm). In this pH region, the reaction, at an optimal concentration of mercurous chloride, gave the stable product while the addition of excess mercurous chloride resulted in the disappearance of the uv absorption. Although in the acidic pH region (pH 1·8–3·6) the reaction product was less stable than in the neutral pH region, it showed the uv absorption independent of a final mercurous chloride concentration of 2×10^{-5}–2×10^{-4} M. Hence

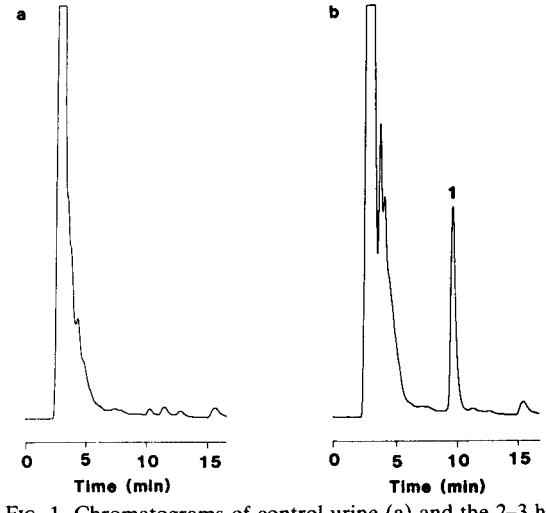


FIG. 1. Chromatograms of control urine (a) and the 2–3 h urine after oral administration of ampicillin (b). Injection volume: 20 μl. Sensitivity: 0·256 aufs. Assignment: peak 1, piperazine-2,5-dione, whose concentration was estimated to be 101 μg ml^{-1}.

* Correspondence.

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.

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Uridine-induced hyperthermia in the rabbit

J. C. CRADOCK*, B. R. VISHNUVAJJALA, T. F. CHIN†, H. D. HOCHSTEIN‡, S. K. ACKERMAN‡, *Pharmaceutical Resources Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20205, †Pharmaceutical Services, College of Pharmacy, The University of Iowa, Iowa City, Iowa 52242, and ‡Center for Drugs and Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, Maryland 20205, USA*

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).

* Correspondence and address: Building 37, Room 6D-12, National Cancer Institute, Bethesda, Maryland 20205, USA.

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