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High-Pressure Liquid Chromatographic Analysis of Antipyrine in Small Plasma Samples

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Abstract □ A high-pressure liquid chromatographic (HPLC) method was developed for the assay of antipyrine in small (0.1-ml) plasma samples using aminopyrine as the internal standard and a reversed-phase microparticulate column. The assay sensitivity (1 μg/ml) permits development of a plasma level–time curve using a single rat. The mean (± *SE*) plasma elimination half-life in rats was 1.28 ± 0.14 hr. A comparison of the spectrophotometric method with the HPLC method yielded a correlation coefficient of 0.98. The HPLC assay for antipyrine is rapid and precise and can be used for hepatic drug metabolism study in a single animal.

Keyphrases □ Antipyrine—analysis, high-pressure liquid chromatography, plasma, rats □ High-pressure liquid chromatography—analysis, antipyrine, plasma, rats □ Analgesics—antipyrine, high-pressure liquid chromatographic assay, plasma, rats

The plasma antipyrine elimination rate is commonly used to study hepatic drug metabolism *in vivo* (1–5). Antipyrine is metabolized almost completely by the liver enzymes (6), and protein binding of the drug is minimal (7).

BACKGROUND

The spectrophotometric assay of Brodie *et al.* (8) generally is used for measuring plasma antipyrine levels. The spectrophotometric method is tedious and may yield high blank values. Furthermore, the method requires 1–2 ml of plasma, which precludes obtaining multiple samples from a small animal such as the rat.

GLC analyses of antipyrine also have been reported (3, 9). Although these methods appear to be more precise than the spectrophotometric method, they still require a 1.0-ml plasma sample and drug extraction. Another assay for plasma antipyrine using ^{14}C-label drug (4) is very sensitive but requires expensive equipment and involves the hazards of radioactivity.

High-pressure liquid chromatography (HPLC) has been applied to the separation and quantitation of many drugs. A sensitive HPLC procedure for antipyrine using a silica gel column was reported (10). This procedure requires 0.5 ml of plasma for drug extraction. A rapid, sensitive method was needed that utilizes smaller blood samples, 0.1–0.2 ml, so that an antipyrine pharmacokinetic profile can be obtained in a single laboratory animal such as the rat. Recently, HPLC procedures utilizing reversed-phase chromatography were developed for drug analysis in biological tissues (11). The advantage of reversed-phase HPLC is that minimal sample cleanup is required prior to chromatography; therefore, smaller tissue samples may be used.

This report describes a rapid HPLC procedure which is more precise than the spectrophotometric method and utilizes small plasma sample volumes.

EXPERIMENTAL

Materials—Antipyrine NF and aminopyrine NF were used as standards[1]. All other reagents, analytical grade or better, were purchased from commercial sources.

Animals—Adult, male, Sprague–Dawley strain rats[2], 200–350 g, were used.

Plasma Assay and Animal Dosing—For standard curves, heparinized blood samples were removed from the abdominal aorta of nonmedicated ether-anesthetized rats, placed into centrifuge tubes, and centrifuged to obtain the plasma fraction. Plasma aliquots (0.1 ml) were pipetted into 1.5-ml microtest tubes[3] and spiked with 10 μl of concentrated antipyrine solutions to yield final drug concentrations corresponding to 0, 1.5, 3, 4.5, 6, 12, 25, and 50 μg/ml of plasma.

To each microtest tube was added 0.05 ml of 20% (w/v) $ZnSO_4 \cdot 7H_2O$ in 50% (v/v) methanol–water containing aminopyrine, 100 μg/ml, as the internal standard. The mixture was mixed on a vortex mixer. Then 0.05 ml of saturated barium hydroxide solution was added to the mixture. The final mixture was vortexed and centrifuged for 10 min. A 10-μl sample of the supernate was removed and subjected to HPLC.

Rats were given 100 mg of antipyrine/kg ip (injection volume of 0.5 ml/200 g). Blood samples of approximately 250–300 μl were removed from the tail vein at 0, 2, 3, 4, 5, and 6 hr. The samples were placed into heparinized microcentrifuge tubes and centrifuged to obtain the plasma fraction. Plasma aliquots, 0.1 ml, were processed along with the spiked plasma standards described.

HPLC—Samples were chromatographed on a high-pressure liquid chromatograph[4] equipped with a universal liquid chromatographic injector, a UV (254-nm) absorbance detector, and a strip-chart recorder[5]. The deproteinated plasma samples (10 μl) were chromatographed at room temperature on a microparticulate[6] reversed-phase HPLC column, 4 mm × 30 cm, with an eluting mobile phase of methanol–water (50% v/v). The mobile phase flow rate was adjusted to 1 ml/min with an inlet pressure of ~1750 psig. The chart speed was 0.25 cm/min.

A standard curve was obtained by comparing the peak height ratio of antipyrine to aminopyrine and the spiked plasma antipyrine concentration. Unknown plasma sample concentrations were calculated by comparing the peak height ratios of the samples to the processed standards.

Correlation with Spectrophotometric Assay—Ten plasma samples obtained from nonmedicated rats were spiked with antipyrine at con-

[1] Merck & Co., Rahway, N.J.
[2] Charles River CD.
[3] Eppendorf, Brinkmann Instrument, Westbury, N.Y.
[4] Model ALC/GPC 204, Waters Associates, Milford, Mass.
[5] Fisher Recordall, series 500, Fisher Scientific Co., Pittsburgh, Pa.
[6] μBondapak C_{18}, Waters Associates, Milford, Mass.

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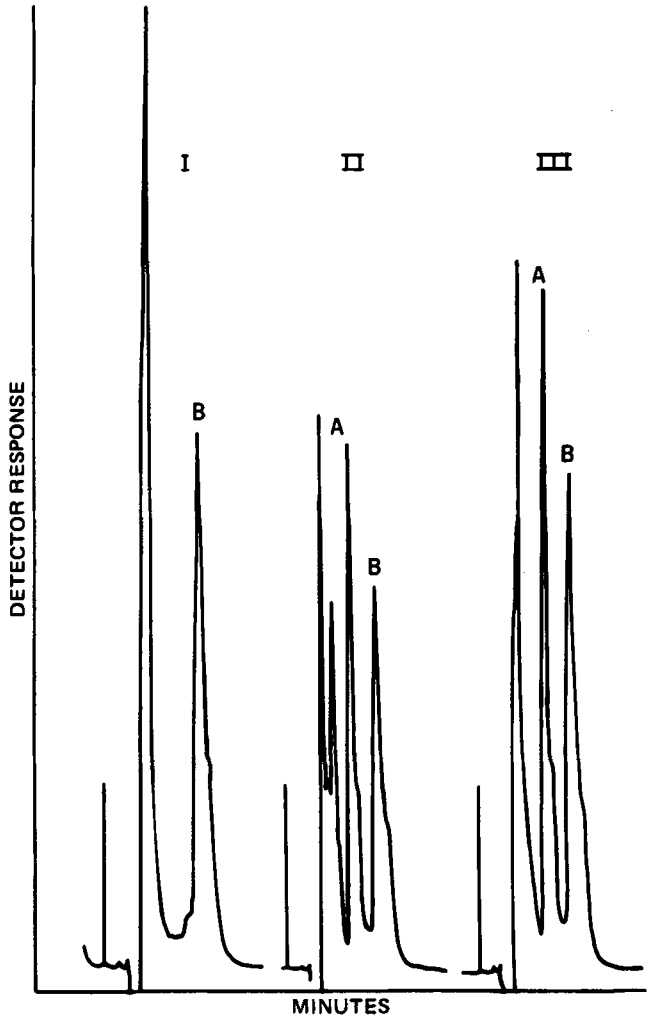

Figure 1—*High-pressure liquid chromatograms of blank plasma with 50 μg of aminopyrine/ml (B) as the internal standard (I), rat plasma collected 2 hr after the intraperitoneal injection of 100 mg of antipyrine/kg (A) containing 50 μg of aminopyrine/ml (B) (II), and processed standards in control rat plasma containing 37.5 μg of antipyrine/ml (A) and 50 μg of aminopyrine/ml (B) (III).*

centrations of 0, 5, 10, 20, and 40 μg/ml of plasma. The samples were split; one portion was assayed by the precipitation procedure of Brodie *et al.* (8), and the second portion was assayed by HPLC. The two assays were compared to obtain a correlation coefficient.

RESULTS

Chromatograms of plasma samples containing antipyrine demonstrated no interfering peaks with normal plasma constituents (Fig. 1). Retention times for antipyrine and aminopyrine were 5.6 and 8.7 min, respectively. Processed standard curves of antipyrine plasma samples were linear to 50 μg/ml, and the assay sensitivity was ~1.0 μg/ml. Standards were processed in triplicate, and the regression line slope was calculated as 27.1 with a correlation coefficient of 0.99, demonstrating excellent linearity. The assay sensitivity was ~1.0 μg of antipyrine/ml of plasma.

A comparison of the HPLC and spectrophotometric assays demonstrated excellent correlation. A correlation coefficient of 0.98 and a slope of 0.97 were obtained.

To demonstrate the application of the HPLC method *in vivo*, three rats were given 100 mg of antipyrine/kg ip. The plasma elimination of antipyrine for each rat was first order (Fig. 2). The mean (± *SE*) plasma elimination half-life for antipyrine was 1.28 ± 0.14 hr, which agrees with the reported value of 85 min (2).

DISCUSSION

The main advantage of the HPLC assay of antipyrine over previous

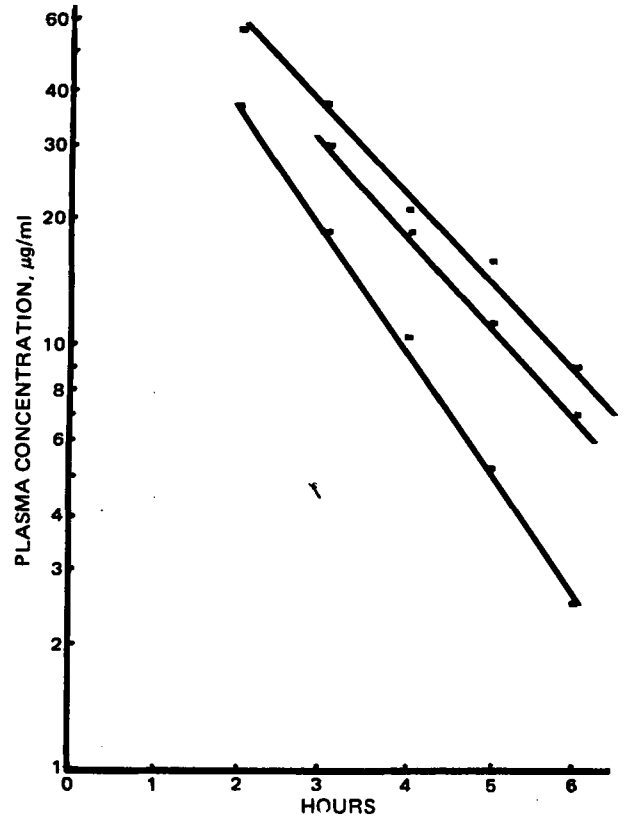

Figure 2—*Plasma antipyrine levels in individual rats. Each rat was given 100 mg of antipyrine/kg ip. Plasma samples were assayed for antipyrine according to the described HPLC procedure.*

methods is that a complete plasma concentration *versus* time curve may be obtained from a single rat. Previously, investigators performing pharmacokinetic and drug metabolism studies *in vivo* with the rat as the animal model needed many rodents to obtain sufficient data points to describe accurately the plasma elimination of antipyrine (2). This micromethod for antipyrine measurement allows the use of the rat as its own control since very small (0.1 ml) plasma samples are used. Although the assay sensitivity is only 1.0 μg/ml, enough data points describing antipyrine elimination kinetics may be obtained quickly and accurately. The assay sensitivity probably could be improved by further sample cleanup and injection of larger sample volumes on the chromatograph.

The *in vivo* data demonstrated that reasonable elimination half-lives for antipyrine may be obtained by this HPLC method. Therefore, this method should be useful for studying the effects of environmental factors of various xenobiotics on hepatic drug metabolism activity in the rat *in vivo* using antipyrine pharmacokinetics as one parameter.

In comparison to the popular spectrophotometric method for antipyrine (8), the HPLC assay is simple, rapid, and precise. Moreover, there is no need for involved separation and purification techniques. Since results of the HPLC method agree well with those of the spectrophotometric method, as shown by a correlation coefficient of 0.98, the HPLC assay offers a reasonable alternative for antipyrine measurement.

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Metabolic Fate of N^6-Benzyladenosine and N^6-Benzyladenosine-5′-phosphate in Rats

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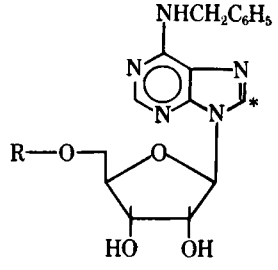
Abstract □ The radiolabeled antitumor nucleoside (^{14}C-8)-N^6-benzyladenosine and its (^{14}C-8)-5′-phosphate were administered to rats intravenously, and their metabolic fate was studied. Twenty-nine percent of the radioactivity was recovered in the 48-hr urine collection after (^{14}C-8)-N^6-benzyladenosine administration. The following metabolites were isolated: unchanged N^6-benzyladenosine (20%), adenine (12%), uric acid (5%), and N^6-benzyladenine (0.3%). In the case of (^{14}C-8)-N^6-benzyladenosine-5′-phosphate, a total of 28% of the radioactivity was recovered in the 48-hr urine collection and the following metabolites were isolated: N^6-benzyladenosine (40%), uric acid (12%), adenine (trace), and unidentified urea derivatives (30%). Metabolism of N^6-benzyladenosine appears to involve N-debenzylation to some extent, followed by conversion to adenine and uric acid. N^6-Benzyladenosine and its 5′-phosphate differ from other adenosine analogs in being retained in significant amounts by the animals.

Keyphrases □ Benzyladenosine—metabolism, rats □ Benzyladenosine phosphate—metabolism, rats □ Antineoplastic agents—benzyladenosine, metabolism, rats

Since N^6-(Δ^2-isopentenyl)adenosine (1) exhibited growth inhibitory activity in mammalian cell lines as well as *in vivo*, several 6-substituted adenosines were synthesized and their antitumor activities were determined (2, 3). Among them, N^6-benzyladenosine (I) was of particular interest since it exhibited a significant growth inhibitory activity against mouse L-1210 leukemia (2). In rats, N^6-benzyladenosine, like N^6-(Δ^2-isopentenyl)adenosine, inhibited the incorporation of precursors into DNA, RNA, and protein (4). More recently, this nucleoside underwent a clinical trial as an antitumor agent (5). Subsequently, because of the poor solubility of N^6-benzyladenosine, a more water-soluble derivative, N^6-benzyladenosine-5′-phosphate (II) (6), was prepared and used instead. Since initial metabolism studies indicated that N^6-benzyladenosine did not behave like other N^6-substituted adenosines (7, 8), investigations were undertaken to determine the metabolic fate of N^6-benzyladenosine and its 5′-phosphate in rats.



I: R = H

II: R = $-P(=O)-(OH)_2$

* = ^{14}C

EXPERIMENTAL

Materials and Methods—(^{14}C-8)-N^6-Benzyladenosine[1] (I) was synthesized by reacting (^{14}C-8)-adenosine[2] (100 μCi, 2 μmoles), adenosine (10 μmoles), benzyl bromide (20 μl), and dimethylformamide at 40° for 24 hr, followed by heating with 14 N ammonium hydroxide (1 ml) and water (2 ml) at 95° for 3 hr (2). The yield was 9.8 μmoles (81.7%), the total radioactivity was 62.3 μCi, and the specific radioactivity was 6.3 μCi/μmole. (^{14}C-8)-N^6-Benzyladenosine-5′-phosphate (II) was synthesized by phosphorylation of (^{14}C-8)-N^6-benzyladenosine, prepared from (^{14}C-8)-adenosine (130 μCi, 10 μmoles), phosphorus oxychloride (50 μl), and triethyl phosphate (1 ml) (6). The yield was 5.1 μmoles (51%), the total radioactivity was 44.93 μCi, and the specific radioactivity 8.81 μCi/μmole.

Chromatography—Paper chromatograms were developed in a descending manner on 3 MM filter paper during initial preparative stages and on acid-washed (No. 1) filter paper when purifications and comparative identification were required[3]. The following solvent systems were utilized for paper chromatography: A, isopropanol–concentrated ammonium hydroxide–water (7:1:2), B, ethyl acetate–2-ethoxyethanol–16% formic acid (4:1:2), upper phase; C, n-butanol–water–concentrated ammonium hydroxide (86:14:5), upper phase; D, n-propanol–concentrated ammonium hydroxide–water (11:2:7); and E, ethyl acetate–n-propanol–water (4:1:2), upper phase. Chromatograms were viewed under a shortwave UV lamp at 254 nm. All UV-absorbing areas were eluted and analyzed in a UV spectrophotometer[4].

Radioactivity Determination—The liquid samples (100–250 μl) were counted for radioactivity in scintillation vials containing 10 ml of the scintillation fluid[5]. The solid samples (feces and organs) were homogenized, aliquots corresponding to 0.5 g were placed in a gelatin capsule and burned in a sample oxidizer[6], and the radioactive carbon dioxide was counted.

Labeled Compound Administration—Two Sprague–Dawley rats (male, 200 g) were given aqueous (^{14}C-8)-N^6-benzyladenosine (0.18 mg,

[1] Aqueous solutions of (^{14}C-8)-N^6-benzyladenosine should be stored frozen at −70° or below. When being kept at −20° for 2 months, 5% of the labeled compound underwent degradation to (^{14}C-8)-N^6-benzyladenine.

[2] Specific radioactivity, 50 mCi/mmole; Schwartz Bio-Research, Orangeburg, NY 10962.

[3] Whatman filter papers were used for paper chromatography.

[4] Beckman Acta V.

[5] Five percent Bio-Solv (BBS-3) solubilizer (Beckman Instruments, Fullerton, CA 92634) in diluted Permafluor III (Packard Instrument Co., Downers Grove, IL 60515).

[6] Packard Tri-Carb model 306. The $^{14}CO_2$ was trapped in Carbo-Solv (Packard Instrument Co.).

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