High-Pressure Liquid Chromatographic Analysis of a Tetrazolylchromone in Biological Fluids

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Received August 17, 1977, from the *Toxicology Department, Miles Laboratories, Inc., Elkhart, IN 46515.* Accepted for publication December 6, 1977. *Present address: J. T. Baker Diagnostics, Bethlehem, Pa.

Abstract □ Biological levels of a tetrazolylchromone were determined by high-pressure liquid chromatography without interference from natural physiological components and metabolic products.

Keyphrases □ Chromone, substituted—high-pressure liquid chromatographic analysis in biological fluids □ High-pressure liquid chromatography—analysis, substituted tetrazolylchromone in biological fluids □ Tetrazolylchromone, substituted—high-pressure liquid chromatographic analysis in biological fluids □ Antiallergic compounds, potential—substituted tetrazolylchromone, high-pressure liquid chromatographic analysis in biological fluids

Substituted chromones have demonstrated potential usefulness as prophylactic adjuncts in management of patients with severe perennial bronchial asthma (1). The sodium salt of 5-(3-*p*-cyanophenoxy-2-hydroxy-1-propoxy)-2-(1*H*-tetrazol-5-yl)chromone[1] (I) is an experimental antiallergic compound whose primary route of administration in humans will probably be inhalation.

For toxicological evaluation of I, an assay was needed for quantitative measurement of unchanged drug in biological fluids. Such an assay was particularly important in inhalation toxicity to establish bioavailability and the dose-dependent nature of drug levels in serum and urine.

Tillman and Whymark (2) reported a spectrophotometric method for the determination of chromones in pharmaceutical preparations. However, this procedure would not be readily adaptable to either biological samples or the potentially large number of samples obtained during a subchronic or chronic toxicity study. TLC was unsuitable as a quantitative tool, and the high molecular weight and variety of polar functional groups precluded the use of GLC.

High-pressure liquid chromatography (HPLC), however, offered the following advantages over other potential methods of analysis: minimal sample preparation, relatively fast separation of the chromone from biological media, and a sensitive absorbance detector. The present report describes the application of HPLC to the analysis of I in biological fluids.

EXPERIMENTAL

Biological Samples—Urine and serum samples obtained during biological disposition studies (3) with 10-mg/kg po or iv doses of ^{14}C-I in rhesus monkeys, beagle dogs, and Charles River COBS-CD rats were assayed radiochemically as well as by HPLC. ^{14}C-I, specific activity of 1.8 μCi/mg, was labeled at the 2-position of the chromone ring and at the tetrazole carbon (asterisks in Structure I).

Apparatus—An HPLC system[2] equipped with a UV detector[3] (254 nm) and attached to a computing integrator[4] was used for the analyses. A 0.45-μm filter system[5] was used to filter mobile phase solvents. A liquid scintillation spectrophotometer[6] was used in radiochemical analyses. Counting was performed for a sufficient time to ensure less than a 2% counting error. All counts per minute were converted to disintegrations per minute after external standardization.

Column—A reversed-phase column[7], 30 × 0.63 cm o.d., with approximately 5000 theoretical plates and a 10-μm nonpolar packing material consisting of a monomolecular layer of octadecyltrichlorosilane was used for the reversed-phase chromatography.

Reagents—Glass-distilled water and methanol[8], the mobile phase solvents, were filtered to remove any particulate matter and to degas the solvents. A paired ion chromatography reagent containing tetrabutylammonium phosphate provided the mobile phase with a large organic counterion (4).

Mobile Phase—One vial of the paired ion reagent[9] was dissolved in 1000 ml of methanol–water (48:52), stirred for 5 min, and filtered through a 0.45-μm filter. The final concentration of the paired ion in solution was 0.005 *M*.

Sample Preparation—Urine, after volume measurement, and serum samples collected at various time intervals were kept frozen until assayed. Prior to HPLC analysis, aliquots of urine samples were centrifuged[10] at 5–10° for 20 min at 10,000 rpm to remove particulate matter. Aliquots (0.5–1.0 ml) of serum samples were pipeted into 12-ml conical-bottom centrifuge tubes. After the addition of equal volumes of methanol, they were mixed on a vortex mixer and centrifuged[10] at 5–10° for 20 min to pack precipitated protein.

In preparation for radiochemical assays, aliquots of urine or serum samples were added directly to the liquid scintillator (5).

Chromatographic Procedure—The mobile phase, containing the paired ion, was set at a flow rate of 1.0 ml/min (inlet pressure of 1400 psig) at ambient temperature. The unattenuated detector signal was fed directly to the computing integrator.

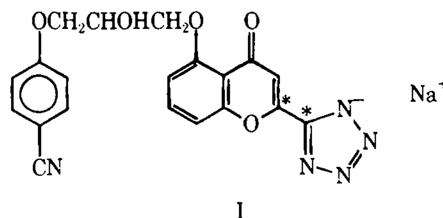
All sample injections (1 μl) were made in triplicate with the flow stopped. Standard samples of I, 2–200 ng/μl, used to establish the standard curve were injected daily to assure that the same detector response and column conditions were maintained. Biological levels of I in standards and samples were calculated using the external standard technique.

Calculations—After samples of I of known concentrations (10 and 30 ng/μl) were injected, the integrator calculated the peak area, based on absorbance, detector response, and a calibration factor (*kf*) using:

$$kf = \frac{\text{area} \times 10^{kf\ \text{exp}}}{\text{concentration}} \qquad \text{(Eq. 1)}$$

After several injections of the standard, a mean *kf* value was calculated and used in Eq. 2 to calculate biological levels of I in unknown samples:

$$\text{concentration} = \frac{\text{area}}{kf \times 10^{kf\ \text{exp}}} \qquad \text{(Eq. 2)}$$



I

[1] Miles Laboratories, Elkhart, Ind.
[2] ALC/GPC 204, Waters Associates, Milford, Mass.
[3] Model 440, Waters Associates, Milford, Mass.
[4] Autolab System I, Spectra-Physics, Santa Clara, Calif.
[5] Millipore Corp., edford, Mass.
[6] Tri-Carb model 3375, Packard Instruments Co., Downers Grove, Ill.
[7] μBondapak C_{18}, Waters Associates, Milford, Mass.
[8] Spectroquality, Matheson, Coleman & Bell, Norwood, Ohio.
[9] PIC Reagent A, Waters Associates, Milford, Mass.
[10] Model RC-2, Ivan Sorvall, Inc., Norwalk, Conn.

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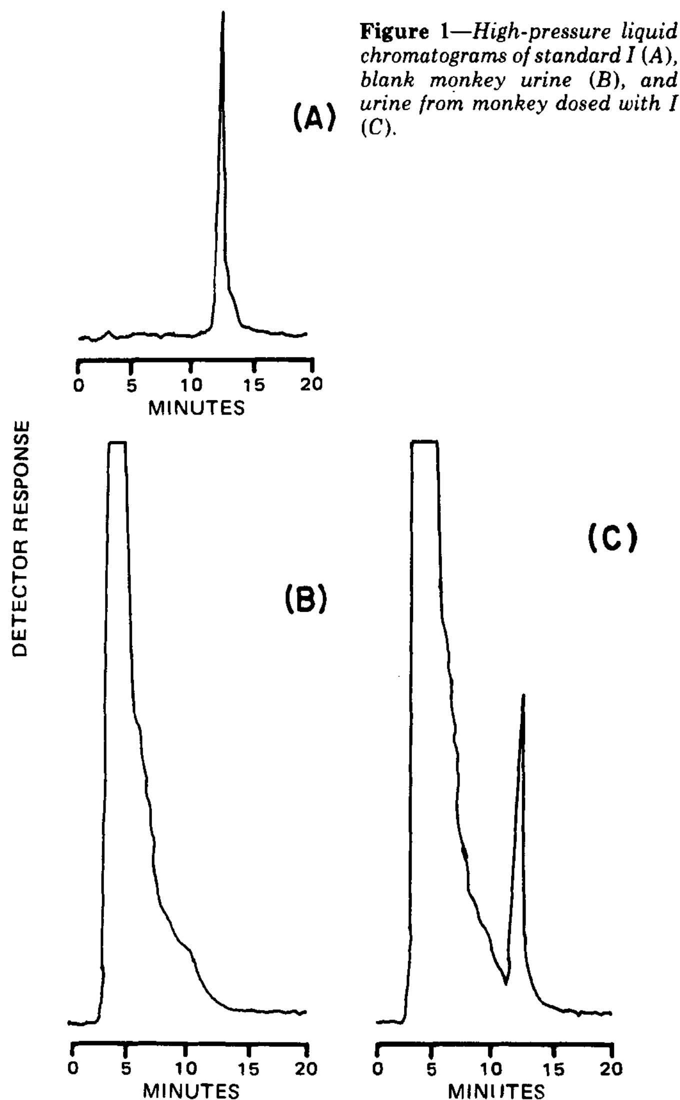

Figure 1—*High-pressure liquid chromatograms of standard I (A), blank monkey urine (B), and urine from monkey dosed with I (C).*

RESULTS AND DISCUSSION

Figure 1 represents typical chromatograms of the standard, urine from undosed monkeys, and urine from monkeys dosed with I. The retention time for I was approximately 11 min. The established techniques separated the chromone from the physiological components of urine and serum from monkeys, dogs, rats, and humans.

The linearity of detector response for I was in the concentration range of 2–200 ng/μl. The sensitivity limit for biological levels of I was 1 ng/μl. The injection technique variation for triplicate 1-μl injections of the same sample was consistently 1–3%. With an appropriate internal standard and/or an automatic injection system, this injection variation could be greatly reduced.

The accuracy of the HPLC method was determined by comparing the computed biological levels of I with those measured by liquid scintillation counting of the same urine and serum samples from monkeys, rats, and dogs dosed with ^{14}C-I. From the biological fluid of each species, the HPLC fraction containing I was collected, analyzed radiochemically, and determined to contain all of the radioactivity of the injected sample. TLC of identical biological samples yielded only one spot. Table I shows that biological concentrations of I, using either method, were not significantly different.

HPLC proved to be a highly sensitive, simple, and fast technique to quantitate I in biological fluids. This method is currently being used to monitor physiological levels of unlabeled I after inhalation of the chromone by monkeys and rats. HPLC can probably be a useful tool for the analysis of other chromone compounds in biological fluids.

Table I—Method Analysis Comparison

Sample[a]	Radiochemical Assay[b], ng Equivalent I/μl	HPLC[b], ng/μl
Dog urine	11.98 ± 0.07	12.02 ± 0.06
Monkey urine	35.71 ± 0.56	36.16 ± 0.11
Rat serum	4.37 ± 0.10	4.18 ± 0.06
Dog serum	3.01 ± 0.08	3.03 ± 0.07

[a] The compound was administered intravenously to dogs and monkeys and orally to rats. [b] The number of replicate assays on the same sample was 10 in the radiochemical system and six in the HPLC system.

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ACKNOWLEDGMENTS

Presented in part at the Federation of American Societies for Experimental Biology, Chicago, Ill., April 1977.

The authors thank Rick Minegar for technical assistance.