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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF VEROFYLLINE IN RAT SERUM

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SUMMARY

A simple and selective high-performance liquid chromatographic procedure has been developed for the determination of verofylline in rat serum. This assay procedure, which is suitable for the analysis of microsamples (0.05 or 0.1 ml serum), involves a single extraction followed by isocratic chromatography on an RP-18 analytical column with ultraviolet detection at 280 nm. The calibration curve is linear within the study range 0.25—75 μg/ml. The intra-day and inter-day coefficients of variation were below 5.7%. The specificity of the assay was confirmed by comparing the lipophilicity, absorbance, and chromatographic property of the apparent and authentic verofylline.

INTRODUCTION

Verofylline [(*d,l*)-3,7-dihydro-1,8-dimethyl-3-(2-methylbutyl)-1H-purine-2,6-dione] is a new polysubstituted methylxanthine bronchodilator. Although its efficacy has been demonstrated both in animals and in man [1–3], neither the pharmacokinetics nor the quantitation of the drug has been reported. To facilitate future pharmacokinetic studies with verofylline, we have developed a reversed-phase high-performance liquid chromatographic (HPLC) procedure for direct determination of verofylline in rat serum.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Waters Model M45 pump, a Waters Intelligent Sample Processor Model 710B, a Waters 440 UV detector with a

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280-nm filter (Waters Assoc., Milford, MA, U.S.A.), and an Ultrasphere Altex (Berkeley, CA, U.S.A.) ODS 5-μm C_{18} column (25 cm × 4.6 mm I.D.). Peak-height integration was done on a Model 3392A Hewlett-Packard integrator (Avondale, PA, U.S.A.).

Chemicals and reagents

Sources of the standard and the internal standard were as follows: verofylline (Berlex Labs., NJ, U.S.A.) and indomethacin (MSD, West Point, PA, U.S.A.). Sodium acetate and glacial acetic acid were of reagent grade and obtained from Sigma (St. Louis, MO, U.S.A.); methanol and methylene chloride were of HPLC grade and obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Verofylline and indomethacin stock solutions were separately prepared in methanol to a concentration of 1 mg/ml and stored at −20°C. Both stock solutions were stable for at least one year. Standard aqueous solutions from 2.5 to 30 μg/ml were obtained through serial dilution of the stock solution with either a buffer or drug-free rat serum. The working solution of the internal standard was 10 μg/ml in methanol.

The mobile phase was 0.5 m*M* sodium acetate buffer (all buffers were adjusted to pH 4.0 with glacial acetic acid unless otherwise indicated)—methanol (30:70). The mixture was filtered and degassed prior to use. The flow-rate was 1 ml/min and HPLC was carried out at room temperature.

Standards and sample preparations

Starting with 0.05 ml of a standard or serum sample, indomethacin was added as the internal standard (0.05 ml). A 3-ml volume of methylene chloride and 0.1 ml of 1 *M* hydrochloric acid were then added. Tubes were shaken for 10 min at medium speed and then centrifuged for 10 min at 1000 *g*. The aqueous layer was aspirated and discarded. The remaining organic layer was then evaporated to dryness in a 35—40°C temperature-controlled bath under a stream of nitrogen gas. The dry residue was redissolved in 0.2 ml of mobile phase; a 0.075-ml aliquot was injected onto the HPLC system.

Calibration and data analysis

The peak-height ratios of verofyllin/internal standard and standard concentrations were used to establish the calibration curve. The regression line was forced through the origin if the range of confidence limits (95%) of the intercept overlapped the origin. The unknown concentrations was calculated directly from the regression line.

Statistical analysis was performed using the paired Student's *t* test. A *P* value of less than 0.05 was considered significant. Regression lines were fitted by the perpendicular least-squares method of Riggs et al. [4] when both variables contained an error.

Test for selectivity

Since the metabolic pathway of verofylline is not yet fully understood, direct verification of metabolite interference is not permitted. The specificity of the assay was tested by manipulating the assay conditions, thereby directly challenging the authenticity of the apparent verofylline.

Comparative distribution ratios [5]. The distribution ratio is defined as:

$$D = \frac{\text{amount in aqueous layer after equilibrium}}{\text{amount in both organic and aqueous layers}}$$

Using an organic solvent with different degrees of polarity, the distribution ratios from spiked verofylline serum (authentic) and in vivo serum sample (apparent) were compared to test assay specificity.

Three Sprague–Dawley rats were given three doses of verofylline (0.75 mg/kg) 4 h apart in order to maximize the accumulation of metabolites. Under light diethyl ether anesthesia, animals were bled from the abdominal aorta. Serum samples were harvested, pooled together and kept frozen at −20°C. Analyses were carried out within three to four days.

Drug-free blood samples were collected from healthy Sprague–Dawley rats and then serum was harvested. Verofylline was spiked into the serum to make a final concentration comparable to that obtained from the medicated rats.

Serum samples (2 ml) from medicated and non-medicated rats were extracted with 5 ml of methylene chloride and 1.0 ml of 1 *M* hydrochloric acid. After a 10-min centrifugation (1000 *g*), the aqueous phase was aspirated off. The organic phase was then evaporated to dryness under a stream of nitrogen gas. The residue was redissolved with 5 ml of buffer (pH 7.4). For each of 0.25 ml of reconstitute, 0.25 ml of hexane with 0–100% methylene chloride were added for extraction. Both before and after extraction, an aliquot of the aqueous layer was directly injected onto the HPLC system. The peak height was used to establish the distribution ratio. Finally, distribution ratios were plotted against the percentage methylene chloride in hexane for the authentic and apparent drug.

Modified absorbance ratio. The selectivity of the assay was challenged by using two different detection wavelengths. The effluent from the HPLC system was monitored simultaneously by 254- and 280-nm UV detectors. Calibration curves were established for each wavelength monitored to calculate the unknown concentration of the samples.

Blood samples were obtained during a verofylline pharmacokinetic study in three Sprague–Dawley rats. Ten blood samples were drawn from each rat through a previously implanted jugular cannula at various times up to 10 h after the intravenous dose. Serum samples were harvested and kept frozen until analysis. Concentrations obtained from two calibration curves were compared with the paired *t* test.

Different HPLC system. The present assay was checked by a different HPLC system. While the extraction procedure remained the same, the column and the pH of the mobile phase were different. It utilized a C_{18} μBondapak 10-μm column, 30 cm × 3.9 mm (Waters Assoc., Milford, MA, U.S.A.) with a pH 8.0 sodium acetate buffer in the mobile phase. These changes reversed the order of elution on chromatography for verofylline and the internal standard.

Similar serum samples obtained as described above were extracted and reconstituted. Then aliquots were injected onto these two HPLC systems. Their concentrations were determined accordingly. The results were compared with the paired Student's *t* test.

Recovery studies. The absolute peak heights of verofylline and the internal standard from extracted and unextracted samples were compared.

RESULTS AND DISCUSSION

Assay

Fig. 1 shows the results of the analysis of verofylline and indomethacin from a serum blank, a standard solution, and a verofylline-treated rat. The chromatogram from a rat blank serum shows no apparent interfering peak. Complete peak resolution was achieved in 9 min from the standard solution and from the rat serum sample. The verofylline peak resolved completely from the unknown metabolite peak as well as from other endogenous peaks in the serum.

The peak-height ratio of verofylline to the internal standard was related to verofylline concentration and was found to be linear within the range 0.25–75 μg/ml. The correlations of regression for the standard curves were 0.997 or better. Table I shows the intra-day and inter-day coefficients of variation of the assay. All coefficients of variation were below 5.7%.

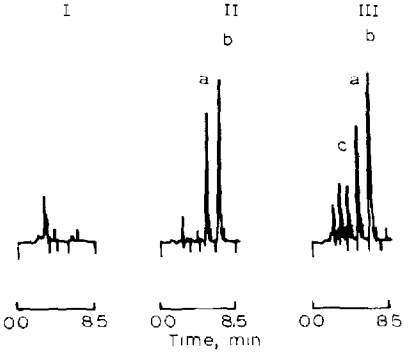

Fig. 1. Chromatograms of rat serum blank (I), standard (II) and rat serum sample (III) obtained after an intravenous dose of verofylline (1 mg/kg body weight). Peaks: a = verofylline; b = internal standard; c = unknown metabolite.

TABLE I

ASSAY VARIABILITY OF VEROFYLLINE

Concentration added (μg/ml)	Concentration found (%)	Coefficient of variation (%)	*n*
Intra-day			
0.25	97.6	5.70	8
2.50	101.0	1.02	8
10.0	97.9	3.80	5
Inter-day			
0.25	96.8	4.63	6
2.50	101.0	2.99	6
10.0	99.4	2.41	6

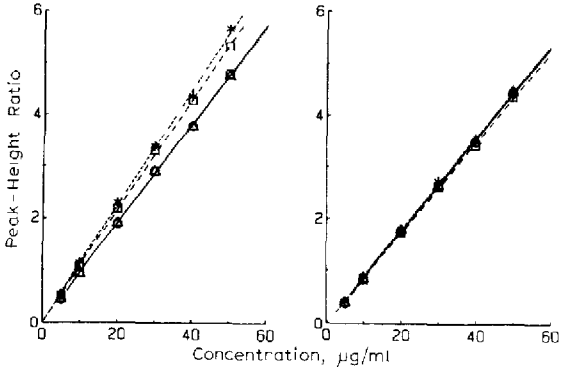

Fig. 2. Calibration curves from water (○), buffer (△), 4% human serum albumin (∗), and rat serum (□) with (right panel) and without (left panel) 1 *M* hydrochloric acid added. Each point represents the mean of duplicates.

Fig. 2 shows the calibration curves of verofylline constructed by the peak-height ratio of internal standard and verofylline standard solution in water, buffer, 4% human serum albumin, and rat serum. There was a distinctive difference in the regression line in these two media. However, when 0.1 ml of 1 *M* hydrochloric acid was added to the extraction procedure, the calibration curves obtained from these media became identical (Fig. 2). It is apparent that the added hydrochloric acid improved the extractability of the compounds.

The advantage of having identical calibration curves from buffer and serum standard solution is obvious. It eliminates the need for preparing another set of calibration curves in situations where samples contain variable protein concentrations such as in measurement of drug—protein binding. This helps to avoid variability within the same experiment.

Selectivity

Comparative distribution ratios. The authenticity of the apparent verofylline was challenged by extractability from solvents of different degrees of polarity. Fig. 3 shows that the distribution ratios (aqueous/organic) of apparent and authentic verofylline decreased in a reverse sigmoidal fashion as the percentage

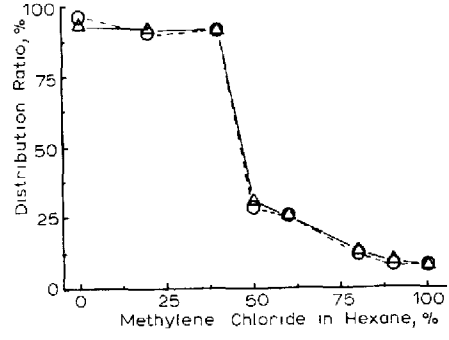

Fig. 3. Distribution ratios of verofylline in aqueous-to-organic phase were plotted against the percentage methylene chloride in hexane. (○) Authentic; (△) apparent verofylline from pooled rat serum after multiple doses of verofylline.

methylene chloride increased. The distribution ratios of the apparent verofylline were superimposed with those of the authentic drug, suggesting that the lipophilic property of the apparent verofylline in rat serum behaves similarly to that of authentic verofylline.

Modified absorbance ratio. Since the effluents of the column were connected to two different UV wavelength detectors in series, the absorbance of apparent verofylline and the internal standard were montiored simultaneously while all other conditions remained the same.

The calibration curves were constructed from standards based on 254- and 280-nm detection. The comparison of the concentration of verofylline samples obtained from a pharmacokinetic study is shown in Fig. 4. The serum concentrations of the apparent verofylline derived from these two standard curves appear to be identical. The slope of the regression line was 1.05 (r = 0.998) with an intercept passing through the origin. A paired t test confirmed that there was no statistical significance between these methods.

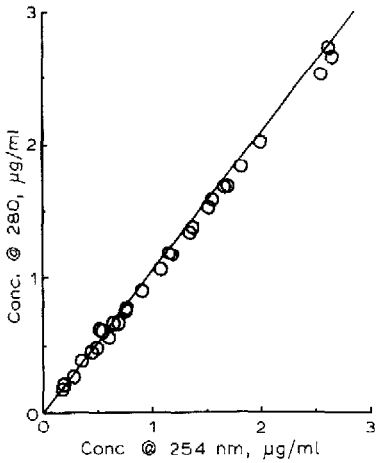

Fig. 4. Comparison of verofylline serum concentration determined from monitoring at 254- and 280-nm wavelengths following HPLC. The slope of the regression line is 1.05 (r = 0.998). See text for details.

Different HPLC system. By changing the pH of the mobile phase and other conditions within the HPLC system, the authenticity of apparent verofylline against that of the standards is directly challenged by its chromatographic property. It is of interest to point out that in altering the pH of the mobile phase, the elution order of the verofylline and the internal standard reversed. At pH 8.0, the retention times of verofylline and indomethacin were 6.6 and 3.9 min, respectively.

With an approach similar to that described above for absorbance ratios, the serum concentrations of verofylline determined from these two HPLC systems were compared (Fig. 5). A linear relationship with an r value of 0.998 was found. The regression analysis gave a slope of unity and zero intercept, suggesting that the apparent verofylline from the rat serum behaved similarly to the authentic verofylline in the standards in these two HPLC systems.

Accurate and specific measurement of the drug of interest in serum is an

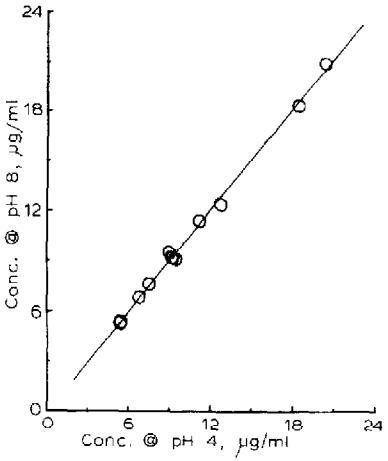

Fig. 5. Comparison of verofylline serum concentration determined from using HPLC mobile phases of pH 4.0 and 8.0. The slope of the regression line is 1.00 (r = 0.998). See text for details.

essential part of any pharmacokinetic study. The present verofylline assay utilized as little as 0.05 ml of rat serum with a single-step extraction followed by a C_{18} column separation. This assay appears to be trouble-free because of its low intra- and inter-day variability throughout the concentration range, its uniform recovery (72—78%), and the ability to use the same calibration curve irrespective of its medium.

The selectivity of this assay has not been tested with identified metabolites. However, using serum from rats with repeated doses of verofylline, its selectivity was directly challenged by lipophilicity, absorbance, and the chromatographic property of verofylline. All of these indicated that the assay was specific.

Although the therapeutic serum concentration range of verofylline in man has not been established, the present assay can be easily modified in clinical applications. The sensitivity of this assay is 50 ng/ml with 0.1-ml sample volume. Improvement in sensitivity may be achieved by simply increasing the volume of the sample.

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