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Note

Analysis of pentoxifylline in rabbit plasma using a Hisep™ high-performance liquid chromatography column

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Pentoxifylline is a hemorrheologic agent used for the management of patients with intermittent claudication and other vascular insufficiencies [1–3]. Administration of pentoxifylline decreases blood viscosity, improves capillary flow, and increases erythrocyte penetration into capillaries [2]. Relative to other methylxanthines, pentoxifylline is a clinically useful drug as it exhibits little influence on the cardiovascular system [3]. Also, in contrast to the use of corticosteriods which increase the likelihood of secondary infections, the administration of pentoxifylline does not cause secondary infections and has actually demonstrated protection from staphylococcal infections in mice [1].

The terminal half-life of pentoxifylline is approximately 1 h in humans, and the clearance of the drug is at least as great as hepatic blood flow. Hence, plasma concentrations of pentoxifylline decrease repidly as a result of several proposed mechanisms of metabolism [1,4]. It is therefore necessary to analyze plasma concentrations of pentoxifylline to properly correlate therapeutically significant concentrations to observed biological responses.

The methods by which pentoxifylline has been analyzed include thin-layer chromatography and colorimetry [5], gas chromatography [6], and several different high-performance liquid chromatography (HPLC) protocols [2,7,8]. These methods all involve relatively large sample volumes, utilize internal standards not readily available, and, most significantly, require lengthy extraction procedures or derivatizations.

Several HPLC methods for pentoxifylline analysis that have recently been suggested reduce the sample treatment and preparation time relative to other works [9,10]. However, these assays require filtration and solvent extractions to remove substances typically found in biological matrices, such as plasma proteins and lipids, which normally interfere with HPLC analysis. The removal of such

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substances is necessary in these instances to prevent precipitation onto column frits and pores, but is time-consuming and can introduce error in the quantitation of an analyte.

This paper describes a method of HPLC analysis of pentoxifylline in rabbit plasma where sample preparation has been virtually eliminated, resulting in a reduction of analysis time and error. The decrease in sample preparation is achieved by utilization of a Hisep™ HPLC column which contains a shielded hydrophobic stationary phase designed to selectively retain small compounds such as drugs. Large, hydrophobic molecules are shielded and elute in the void volume rather than precipitate in the column. Thus, plasma samples can be injected into the column for drug analysis with minimal sample perturbation. Other useful considerations of this assay are the small sample size employed (100 μl), the low limit of detection (3.95 nM), and the excellent drug recovery (100.08% average). These factors compare favorably to previously reported measurements for other methods [2,7–10].

In this analysis, rabbit plasma spiked with various amounts of pentoxifylline and acetophenone is eluted from a Hisep chromatography column. The resultant standard curve is analyzed using a weighted linear least-squares treatment where the weighting factor is the inverse of the variance [11,12]. The error found in the regression alalysis, time efficiency of this method of sample preparation, and the drug recovery achieved using this protocol are discussed.

Also demonstrated is the use of this method in a study of pentoxifylline concentrations found in rabbit plasma. For this study, pentoxifylline is administered intravenously in a single bolus dose to rabbits and samples of blood taken over time. The blood is first centrifuged to remove erythrocytes and then treated similarly to the spiked plasma standard used for the standard curve.

EXPERIMENTAL

Reagents and materials

Pentoxifylline and acetophenone were obtained from Sigma and used as received. The water used was purified by the Milli Q water system (Millipore, Bedford, MA, U.S.A.). The acetonitrile and methanol used were HPLC grade from Fisher Scientific (Houston, TX, U.S.A.).

Standard and sample preparation

For the calculation of the standard curve, varying amounts of a 10 μM stock solution of pentoxifylline and 20 μl of a 172 μM stock solution of the internal standard acetophenone were added to 100-μl aliquots of rabbit plasma. Purified, filtered water was added to make the standards equal in volume. The tubes were vortex-mixed followed by the addition of 300 μl of methanol for deproteinization purposes. The resultant standards contained final concentrations of pentoxifylline ranging from 1.92 nM to 3.85 μM and 6.61 μM acetophenone. The tubes

were again vortex-mixed, then centrifuged for 5 min at 1000 *g* at ambient temperature, as suggested by the column manufacturers for plasma injections. The supernatant was separated from the pellet and injected into the sample loop.

The non-plasma standards, used to determine drug recovery in the plasma standards, were made in a similar manner to the plasma standards except that aliquots of 100 μl of water were used instead of rabbit plasma. The range of concentrations of pentoxifylline, the concentration of the internal standard, and the final total volumes were the same as for the plasma standards.

Application

An experiment using two adult New Zealand white rabbits was performed where the rabbits were given a dose of 70 mg of pentoxifylline over a 15-min period. Blood samples obtained in 0.5-h intervals were centrifuged immediately to remove erythrocytes and then placed in a −80°C freezer. The plasma was then subjected to the protocol used for the plasma standards above except no stock pentoxifylline was added, and water was added in amounts such that the final volume was equal to all samples examined.

Chromatography

A Rannin Rabbit (Woburn, MA, U.S.A.) HP syringe pump was used in series with a Waters (Houston, TX, U.S.A.) Model 441 detector with a 280-nm filter and 1-cm optical pathlength. A Rheodyne Model 7125 injector equipped with a 50-μl sample loop was loaded with samples using the filled-loop technique to insure uniformity of injections.

The Hisep column and guard column were prepared by Supelco (Bellefonte, PA, U.S.A.) and had shielded hydrophobic stationary phases with 5 μm diameter. The analytical column was 15 cm × 4.6 mm I.D. Isocratic elution was used with a mobile phase of acetonitrile–water (10:90, v/v). The mobile phase was filtered through a 0.45-μm nylon filter and purged with helium prior to use.

Drug recovery

To determine the drug recovery of the assay, the ratio of chromatographic peak areas of pentoxifylline to that of the internal standard found in plasma samples was compared to the same ratio of the non-plasma standards. Drug recovery (%) is defined as (peak-area $\text{ratio}_{\text{plasma}} \times 100$)/(peak-area $\text{ratio}_{\text{standard}}$).

RESULTS AND DISCUSSION

When prepared according to the assay described above, the plasma samples appeared turbid. However, there was no indication that precipitation occurred in the column as the pressure remained constant at 17.2 bar throughout the experiments and no unexplainable ghost peaks appeared in the analysis. Fig. 1 illustrates typical chromatograms of blank rabbit plasma, a rabbit plasma sample

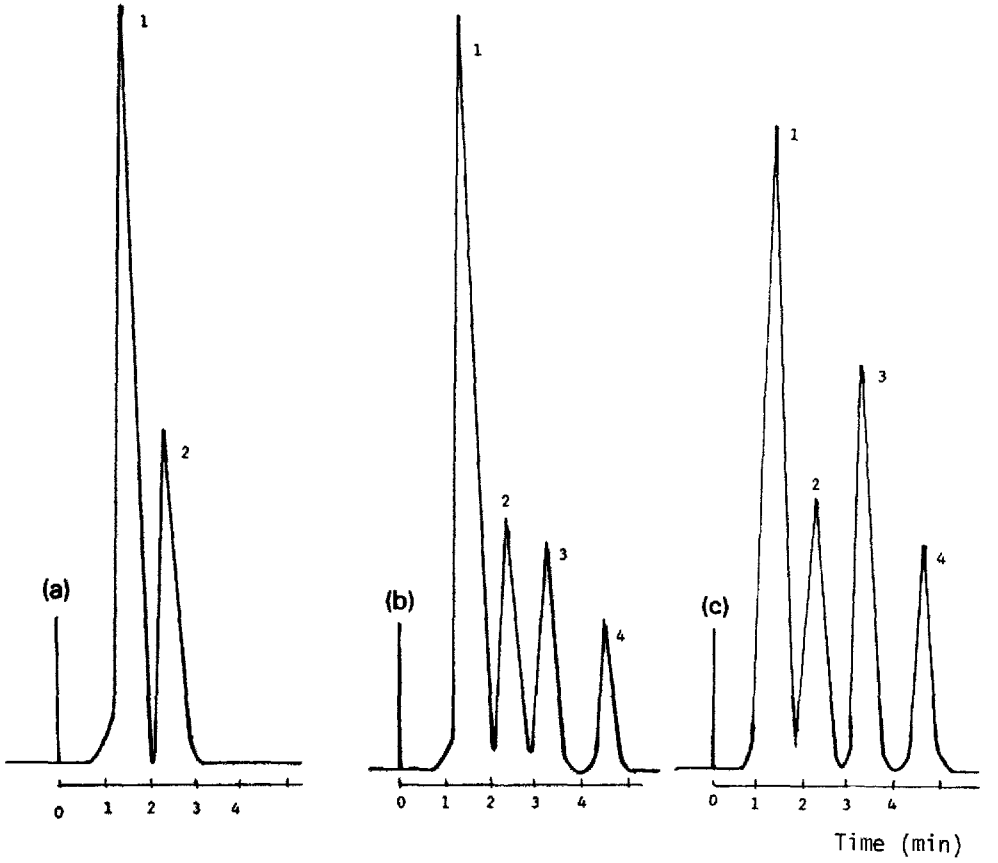

Fig. 1. Chromatograms of (a) blank rabbit plasma, (b) rabbit plasma spiked with pentoxifylline and internal standard, and (c) rabbit plasma sample obtained after intravenous injection. Peaks 1 = void volume; 2 = plasma peak; 3 = pentoxifylline; 4 = acetophenone, the internal standard.

TABLE I

RECOVERY OF PENTOXIFYLLINE FROM RABBIT PLASMA AND LIMIT OF DETECTION OF THE HISEP COLUMN ASSAY

Compound	Recovery[a] (%)			Limit of detection[b] (n*M*)
	1.92 μM	19.2 n*M*	Average	
Pentoxifylline	100 08	98.57	100.08	3 95
Acetophenone	–	–	94.35	–

[a] Drug recovery, as defined in text.

[b] Signal-to-noise ratio = 2.5.

TABLE II

STANDARD CURVES OF PLASMA STANDARDS AND NON-PLASMA STANDARDS FOR PENTOXIFYLLINE ANALYSIS

The results are from weighted linear least-squares treatment of data and used for comparison between plasma and non-plasma standards

Standard	A_1[a] (mean ± S.E.)	A_2[a] (mean ± S.E.)	r^2[b]
Plasma	2.614 ± 0.339	0.5149 ± 0.0050	0.9667
Non-plasma	4.103 ± 0.618	0.5455 ± 0.0068	0.9702

[a] Coefficients for the line $y = A_1 + A_2 x$, where y represents peak-area ratio as described above and x represents pentoxifylline concentration

[b] Square of the correlation coefficient.

spiked with pentoxifylline and with internal standard, and a rabbit plasma sample obtained after injection of pentoxifylline spiked with internal standard. All samples were treated as described above with a methanol addition and a single centrifugation for 5 min. The total time for sample and standard curve preparation was of the order of 30 min. Each sample then eluted in 5 min. Therefore the time conservation of this assay is significant. The amount of plasma required is 100 μl per sample which is also an advantage of this assay.

The drug recovery was calculated as described above and the results are listed in Table I. The average pentoxifylline recovery from the plasma samples as compared to the non-plasma samples is 100.08%. Table I also shows that the average recovery of the internal standard in plasma relative to the non-plasma standards was found to be 94.35%. Total recovery of analytes was expected due to the nature of the assay using this column which includes little sample perturbation and no plasma interference. The limit of detection for pentoxifylline as listed in Table I is 3.95 nM using the criterion of signal-to-noise ratio of 2.5.

The plasma standard curve was analyzed using a weighted linear least-squares analysis with weighting factor equal to the inverse of the variance. Table II lists the equation of the line for this standard curve as well as for the identical study using no plasma. The precision in the analysis is described by the standard error of the slope, also listed in Table II, and is approximately 1% of the value of the slope for both the plasma and non-plasma standards. By comparing the results and error found in each of the two analyses, it can be seen that no significant difference exists suggesting that the plasma caused no interference in the quantitation of pentoxifylline.

An application of the assay was performed by injecting two rabbits with a dose of 70 mg of pentoxifylline in 6 ml of buffer. Blood samples were drawn over time intervals and analyzed for pentoxifylline concentrations. Results are listed in Table III which includes the amount of pentoxifylline found at indicated times

TABLE III

APPLICATATION OF THE ASSAY USING A HISEP CHROMATOGRAPHY COLUMN TO THE ANALYSIS OF PLASMA AFTER INTRAVENOUS INJECTION OF 70 mg OF PENTOXIFYLLINE INTO RABBITS

Blood samples were taken over time and the results were determined using the plasma standard curve.

Time (min)	Concentration of pentoxifylline (mean ± S.E.M.) (μM)
30	4 392 ± 0.0790
60	2 010 ± 0 0100
90	1 375 ± 0 00542
130	1.014 ± 0.0127

determined by interpolation of the standard curve resulting from the plasma standards. The coefficients of the line resulting from the regression of the natural log of concentration of pentoxifylline onto time were calculated, and the equation for this line is $y = 1.892 \pm 0.0766 - 0.02141 \pm 0.000998x$ ($r^2 = 0.9685$). The linear relationship between the natural log of concentration and time indicates that the relationship between concentration and time is exponential as expected. The half-life of pentoxifylline in rabbits calculated from these data is approximately 35 min.

These results demonstrate that the assay works very well in this experiment and that the samples are minimally perturbed.

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