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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FUSIDIC ACID IN PLASMA

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SUMMARY

Fusidic acid was determined in plasma by a high-performance liquid chromatographic method. Fusidic acid was extracted from plasma with acetonitrile that was salted out with ammonium sulfate. Prior to salting out cadmium sulfate was mixed with the acetonitrile–plasma mixture to help remove interfering constituents. A 150 mm × 4.6 mm column packed with 5-μm cyanopropyl stationary phase was used for chromatography. The mobile phase was acetonitrile–20 m*M* sodium dihydrogenphosphate (pH 3.50) (39:61, v/v). An ultraviolet–visible detector was set at 204 nm. The presence of water in the injection solvent had a significant effect on the fusidic acid peak height. A number of clinically important acquired immunodeficiency syndrome drugs did not interfere with the fusidic acid determination. The relative standard deviation varied between 0.99 and 7.8%. A limit of detection of 200 ng/ml was obtained for a 80-μl injection.

INTRODUCTION

Fusidic acid is a steroid antibiotic with an antistaphylococcal activity [1,2]. The chemical structure of fusidic acid is shown in Fig. 1. A recent report indicated the potentiality of fusidic acid therapy for acquired immunodeficiency syndrome (AIDS) patients [3]. With the anticipation of successful use of fusidic acid in AIDS treatment, a rapid and sensitive method is required to monitor the drug level in biological fluids to optimize the treatment and minimize any side-effects.

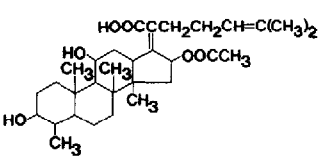

Fig. 1. Chemical structure of fusidic acid.

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Various methods have been developed for the quantitation of fusidic acid. These employed microbiological procedures [4,5], colorimetry [6,7], spectrophotometry [8] and high-performance liquid chromatography (HPLC) [9,10]. However, of all these methods only two used a biological fluid as the sample medium [5,10]. The microbiological procedure [5] was time-consuming and required special technical assistance. The HPLC technique developed by Hikal [10] involved extraction of fusidic acid from plasma with methylene chloride and time-consuming evaporation of the organic solvent. The method was found to have a detection limit of 500 ng/ml in plasma.

It was the main object of this research to develop a more rapid and sensitive HPLC method for the determination of fusidic acid in plasma.

EXPERIMENTAL

Equipment

The chromatographic system consisted of a Waters M-6000A solvent delivery pump (Waters Assoc., Milford, MA, U.S.A.). A Rheodyne Model 7125 injector (Cotati, CA, U.S.A.) with a 200-μl sample loop was used as the injection port. A Kratos Spectroflow 773 variable-wavelength UV–visible detector (Ramsey, NJ, U.S.A.) was used. To record the chromatogram, a Houston Instrument Model 4511 strip chart recorder (Austin, TX, U.S.A.) was used. An Econosphere 150 mm$\times$4.6 mm, 5 μm CN column was purchased from Alltech (Deerfield, IL, U.S.A.). A 2 cm $\times$ 2 mm guard column packed with 8-μm cyanopropyl stationary phase (Upchurch Scientific, Oak Harbor, WA, U.S.A.) was also used. A Sybron-Barnstead 60209 water purification system (Boston, MA, U.S.A.) was used to prepare the deionized water. A Sorval GLG-1 centrifuge from Ivan Sorvall (Norwalk, CT, U.S.A.) was also used. An ultrasonic water bath from L & R Manufacturing Industry (Kearny, NJ, U.S.A.) was used to degas the mobile phase. Volumetric transfers in the range 100–1000 μl were made by using an Eppendorf digital pipette from Cole Parmer (Chicago, IL, U.S.A.).

Materials

The sodium salt of fusidic acid was obtained from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile and methanol were HPLC grade and were obtained from Curtin Matheson Scientific (Houston, TX, U.S.A.). Ammonium sulfate was ACS grade and was obtained from Alfa Products (Danvers, MA, U.S.A.). Analytical-grade sodium dihydrogenphosphate was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Crystalline cadmium sulfate was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). 3′-Azido-3′-deoxythymidine (AZT), 1-chloro-2,4-dinitrobenzene, indomethacin, D-penicillamine and ribavirin were obtained from Sigma (St. Louis, MO, U.S.A.). Imuthiol was obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.). Seven different plasma samples were obtained from Milwaukee Blood Center (Milwaukee, WI, U.S.A.). When not in use the plasma samples were kept frozen at $-10\,^{\circ}$C.

Procedure

Preparation of the stock solution. A stock solution of fusidic acid was made by dissolving 25 mg of sodium fusidate in 3.0 ml of methanol. The solution was then diluted to 250 ml with acetonitrile. The resulting solution of fusidic acid had a concentration of 100 μg/ml. The solution was kept in a freezer at −10°C and brought to room temperature during the experiment period.

Extraction of fusidic acid from plasma. Standard solutions of fusidic acid in plasma were prepared in the following way. To a test tube was transferred 1.0 ml of thawed plasma. The required volume of the fusidic acid stock solution was added to the plasma and the mixture was vortex-mixed for 10 s. The nine standard solutions thus prepared had fusidic acid concentrations of 0.0, 0.3, 1.0, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 μg/ml of plasma. In a typical extraction, the necessary volume of acetonitrile was added to a standard solution to make the total volume of acetonitrile equal to 1.0 ml. The mixture was vortex-mixed for 30 s and centrifuged at 1200 *g* for 2 min. At this point most of the proteins had precipitated. The supernatant was transferred to another test tube and approximately 50 mg of cadmium sulfate were added. The mixture was vortex-mixed for 30 s and centrifuged for 2 min. The supernatant was collected in a second test tube. To this excess ammonium sulfate was added together with vortex-mixing for 30 s and centrifuging for 2 min. At this stage two liquid phases were obtained. A 320-μl portion of the organic layer was transferred to a test tube and 680 μl of water were added to it. The mixture was vortex-mixed for 10 s and 80 μl of the solution were injected into the chromatograph. The entire extraction process took about 10 min.

Preparation of non-extracted solutions of fusidic acid. Standard solutions of fusidic acid were made in acetonitrile–water (32:68, v/v) in the following way. The required volumes of fusidic acid stock solution were added to nine different volumes of acetonitrile to give a total volume of 1.0 ml each and concentrations of 0.0, 0.3, 1.0, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 μg/ml. The solutions were vortex-mixed separately and 320 μl of each of those were mixed with 680 μl of water.

Chromatographic conditions. The mobile phase was prepared by mixing 39 volume percent of acetonitrile with 61 volume percent of 20 m*M* aqueous sodium dihydrogenphosphate. The pH of the aqueous solution was adjusted to 3.50 by adding 85% phosphoric acid. The mobile phase was degassed for 15 min by using an ultrasonic water bath. It was then passed through the analytical column at a flow-rate of 1.0 ml/min. After the complete elution of the fusidic acid peak the column was flushed by the mobile phase at a flow-rate of 4.0 ml/min for at least 12 min. This was done only when plasma samples were injected. The UV–visible detector was set at 204 nm with its sensitivity varying between 0.005 and 0.10 a.u.f.s.

Study of the effect of injection solvent strength on fusidic acid peak height. Fusidic acid was dissolved in water–acetonitrile from 0:100 to 95:5 (v/v). The concentration of fusidic acid was kept constant to 5 μg/ml. Of these solutions, 50 μl were then injected into the chromatograph and their corresponding peak heights were measured.

RESULTS AND DISCUSSION

A typical chromatogram of plasma spiked with fusidic acid is shown in Fig. 2a. The retention time of fusidic acid was found to be 7.7 min. The fusidic acid peak was found to be well resolved from the endogenous peaks of plasma. To show that no interfering peak was present at this position, seven different control plasma samples were chromatographed using the extraction procedure. None of these plasma samples showed any peak at the fusidic acid peak position. The chromatogram of such a blank plasma sample is shown in Fig. 2b.

Fusidic acid concentration was measured by using a calibration curve of peak height versus concentration. The concentration range of fusidic acid in plasma was kept between 0.3 and 50 μg/ml. This range was assumed to be practical since a kinetic study of plasma fusidic acid disappearance showed the peak concentration to be 10.3 μg/ml for a normal dose of 500 mg [10]. However, in the event of administration of higher dosages the calibration range could be reset.

Two different calibration curves were obtained, one with extracted plasma samples and one with non-extracted solutions of fusidic acid. The statistical data for these curves are given in Table I. The curves were found to be linear with a correlation coefficient of 0.999. At the 95% confidence interval, t-value calculation showed that the slope for plasma (curve A) was different from the slope for acetonitrile (curve B). The extraction efficiency of fusidic acid from plasma was $97 \pm 1\%$. The extraction efficiency was calculated from the ratio of the slopes obtained from the calibration curve for plasma and from that of the non-extracted standards.

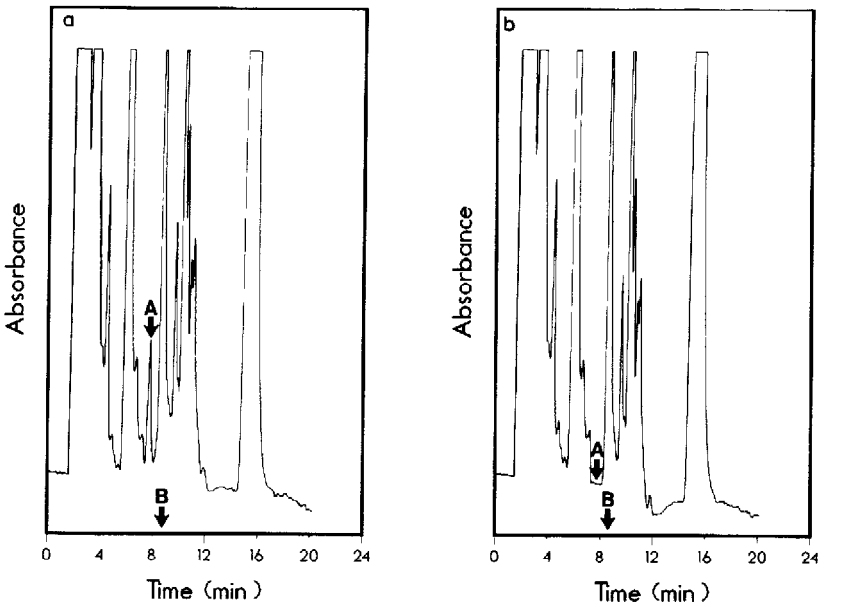

Fig. 2. Chromatograms of an extract of (a) plasma spiked to give 1.0 μg/ml fusidic acid and (b) control plasma. A = fusidic acid peak location; B = time at which the flow-rate was increased to 4.0 ml/min. Detector: 0.005 a.u f.s.

TABLE I

LINEAR REGRESSION ANALYSIS OF CALIBRATION CURVES

$y = mx + b$ where y is the peak height in absorbance units and x is concentration in μg/ml. For both curves $r = 0.999$, $n = 9$.

Curve	Fluid extracted	Slope (mean ± S.D.) ($\times 10^3$)	Intercept (mean ± S.D.) ($\times 10^3$)	Identity of slopes* t_{calc}
A	Plasma	1.41 ± 0.01	−0.151 ± 0.197	4.877
B	No extraction	1.46 ± 0.01	−0 106 ± 0.160	

*$t_{table} = 2.145$, 95%.

TABLE II

REPRODUCIBILITY OF THE METHOD

Actual concentration (μg/ml)	Within-day assay		Between-day assay	
	Concentration (mean ± S.D., $n = 5$) (μg/ml)	R.S.D. (%)	Concentration (mean ± S.D., $n = 5$) (μg/ml)	R.S.D. (%)
0.3	0.387 ± 0.013	3.4	0.373 ± 0.029	7.8
1.0	1.07 ± 0.03	2.8	1.05 ± 0.03	2.9
5.0	5.03 ± 0.10	2.0	4.98 ± 0.07	1.4
10.0	9.76 ± 0.12	1.2	9.98 ± 0.29	2.9
20.0	20.1 ± 0.6	3.0	19.0 ± 0.5	2.6
30.0	30.4 ± 1.0	3.3	29.6 ± 1.0	3.4
40.0	39.9 ± 0.7	1.8	39.5 ± 1.6	4.1
50.0	50.7 ± 0.5	0.99	50.6 ± 1.0	2.0

The method developed was reproducible for both within-day and between-day assays. The results for eight different concentrations of fusidic acid are shown in Table II. For each concentration five determinations were done. The relative standard deviation (R.S.D.) varied between 0.99 and 3.4% for within-day determination and between 1.4 and 7.8% for between-day determination.

A stability study of fusidic acid was performed at room temperature. A 20-ml portion of a plasma sample was spiked with approximately 1 mg of sodium fusidate. The concentration of fusidic acid in the plasma was then determined at various time intervals for 48 h. The concentrations of fusidic acid determined were found to be randomly distributed with an R.S.D. of 1.0%. This indicated high stability of the drug in plasma for at least 48 h.

A series of clinically important drugs used for the treatment of AIDS and AIDS-related complexes was studied for their possible chromatographic interference with the fusidic acid peak. The study was made with the anticipation of the use of fusidic acid with AIDS patients who might also ingest these drugs. Table III lists the drugs studied. None of these drugs was found to interfere with fusidic

acid determination. The retention times and extraction efficiency of these drugs were also calculated. The extraction efficiency was calculated using water as the sample medium. For both penicillamine [11] and imuthiol [12] no peak was observed. The absence could have been the result of a weak chromophoric group. Both AZT [13] and ribavirin [14] eluted very near the void volume because of their polar character. Because of their high polarity both drugs showed poor extraction into the acetonitrile layer. Although 1-chloro-2,4-dinitrobenzene [15] and indomethacin [16] showed excellent extraction efficiency from water (99%) both drugs eluted at least 2 min earlier than fusidic acid.

In the method developed, the fusidic acid after extraction was dissolved in acetonitrile. Acetonitrile as a stronger solvent than the mobile phase produces a leading (asymmetry factor <1) fusidic acid peak when injected. The phenomenon was observed by others and us [17,18] for different injection solvents. This peak distortion gave a poor sensitivity and a loss in resolution from plasma peaks due to band spreading. Water was added to acetonitrile to minimize this injection strength effect. The volume of water added was chosen to achieve the highest sensitivity (tallest peak) and optimum resolution. Fig. 3 shows the relation of peak height of fusidic acid to the volume percent of water in the injection solvent. For all the points, the amount of fusidic acid injected was kept constant (250 ng). As the percentage water was increased the peak height increased and reached a maximum when the volume percent of water was between 68 and 80, after which a further increase in water content decreased the peak height (by 19% at 95% of water). Hence care should be taken in choosing the proper mixture of acetonitrile and water.

The fusidic acid peak was found to be insensitive to the ionic strength of the mobile phase. A change in the concentration of sodium dihydrogenphosphate in the mobile phase (5 to 50 m*M*) did not change the retention time or asymmetry factor of the fusidic acid peak. The pK_a of fusidic acid was reported to be 5.35 [19]. To elute the fusidic acid predominantly in its neutral form the pH of the 20 m*M* sodium dihydrogenphosphate aqueous solution was kept at 3.50. The mobile

TABLE III

LIST OF DRUGS USED TO STUDY THEIR POSSIBLE CHROMATOGRAPHIC INTERFERENCE WITH FUSIDIC ACID

All the chromatographic conditions were kept constant For all drugs extraction was done from water as the sample media.

Drug used	Retention time (min)	Extraction efficiency (%)
AZT	2.2	47
1-Chloro-2,4 dinitrobenzene	5.2	99
Imuthiol	No peak was observed	
Indomethacin	5.7	99
Penicillamine	No peak was observed	
Ribavirin	1.6	3

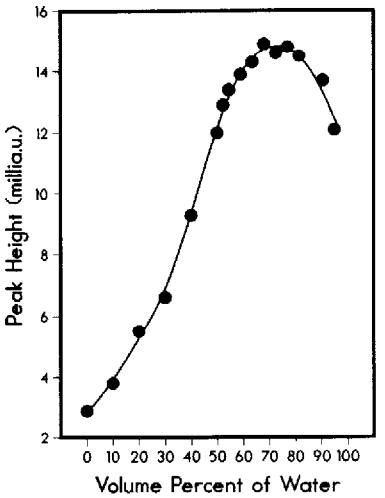

Fig. 3. Peak height of fusidic acid at different volume percentages water in the injection solvent For all points, injection volume and injection amount were 80 μl and 250 ng, respectively.

phase composition used in this method gave the best resolution and reasonable sensitivity. An increase in the acetonitrile content in the mobile phase gave a higher sensitivity, but loss in resolution occurred due to early elution of the fusidic acid peak. On the other hand a decrease in the acetonitrile composition in the mobile phase decreased both resolution and sensitivity. The fusidic acid peak eluted at a later time with band spreading. In addition several interfering peaks appeared at its position.

It was very important that all plasma peaks were eluted before the injection of a new sample. It required at least 45 min for the elution of all the peaks when the mobile phase was passed at a flow-rate of 1.0 ml/min. For a rapid elution of the peaks and a decrease in total chromatographic time, the flow-rate was increased to 4.0 ml/min after the elution of fusidic acid peak. Thus only about 20 min were required between injections. Figs. 2a and b show the point where the flow-rate was increased. Even with the flow-rate change there was ample time between injections for extraction of the next sample. The method thus developed has a limit of detection of 200 ng/ml at a signal-to-noise ratio of 2.

The extraction process developed played a significant role because of the low wavelength maximum of absorption for fusidic acid (204 nm, $\epsilon = 9900$) [19]. At this wavelength almost all compounds with a chromophore would absorb. This required a thorough clean-up of the plasma sample to reduce interfering peaks. Both acetonitrile and methanol were found to precipitate proteins from plasma. Also for both these organic solvents fusidic acid showed similar extraction efficiency from water. However plasma extraction with methanol introduced several interfering peaks in the chromatogram. A further clean-up of the plasma sample was performed by cadmium sulfate. This treatment removed several interfering peaks from the chromatogram.

Different acidic, basic and neutral salts were also studied to find one which would give efficient extraction together with a clean chromatogram. Basic salts like potassium carbonate and disodium hydrogenphosphate gave no extraction of

fusidic acid in acetonitrile. Sodium and potassium chloride showed poor extraction efficiency (<50%). The only salts of choice were acidic salts of which ammonium sulfate and sodium dihydrogenphosphate were found to separate the acetonitrile phase from the aqueous phase. Finally, ammonium sulfate was used as the salt in the extraction process since sodium dihydrogenphosphate removed few interfering peaks.

CONCLUSION

The HPLC method developed for fusidic acid was simple and more rapid than the microbiological procedure [5]. The typical assay time was 30 min. The method described here was more sensitive than the previously reported HPLC technique for fusidic acid [10]. The analytical column showed no significant deterioration even after several hundred injections. Since fusidic acid is highly stable in plasma at room temperature, immediate analysis is not required after the blood is drawn. Because some special drugs used in AIDS did not show any chromatographic interference, the method can be used to monitor the fusidic acid level in patients who would receive those drugs.

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