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SPECTROFLUORIMETRIC DETERMINATION OF DIPYRIDAMOLE IN SERUM — A COMPARISON OF TWO METHODS

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SUMMARY

Two spectrofluorimetric methods for the determination of dipyridamole in plasma are described. The thin-layer chromatographic—fluoridensitometric method utilizes 1 ml of plasma which is extracted at pH 10 with diethyl ether—dichloromethane (80:20). The organic phase is evaporated to dryness, reconstituted in 250 μl dichloromethane and 5 μl are spotted on a silica gel 60 plate. The plate is developed in ethyl acetate—methanol—ammonia (85:10:5), dried, dipped in a paraffin wax solution, dried, and scanned using 380 nm as excitation wavelength, a 430 nm cut-off filter, and collecting all emitted light on the photomultiplier. Quantitation was done by the external standard method, peak heights being measured and a calibration graph constructed. For the spectrofluorimetric method 1 ml of plasma is extracted at pH 10 with 8 ml of hexane—isoamyl alcohol (95:5) and the organic phase used directly for the measurement of the fluorescence intensity (excitation 405 nm, emission 495 nm). Quantitation was done by measuring the fluorescence of standards that were treated as above and constructing a calibration graph of concentration versus fluorescence intensity. Concentrations of unknowns were found by interpolation from this graph. The two methods were found to exhibit good correlation but the spectrofluorimetric method proved to be more amenable to the analysis of a large number of samples.

INTRODUCTION

When asked to perform a bioavailability study on tablets containing dipyridamole (Fig. 1) a review of the literature revealed that only a few analytical procedures for this drug had been published [1—4]. None of these methods suited our particular needs and an analytical method for the determination of dipyridamole in plasma had to be developed. Due to the highly fluorescent nature of this drug, it was decided to utilize this property as a means of determination.

This paper describes two fluorimetric procedures for the determination of dipyridamole in plasma; namely, a thin-layer chromatographic (TLC)—fluoridensitometric method, and a spectrofluorimetric method where the fluores-

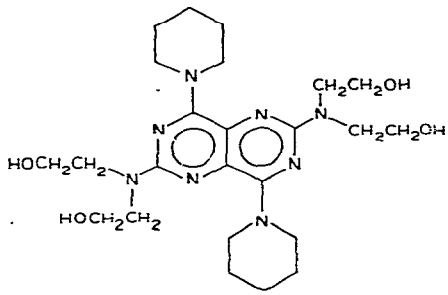

Fig. 1. Chemical structure of dipyridamole [2,6-bis(diethylamino)-4,8-dipiperidinopyrimido-(5,4-*d*)-pyrimidine].

cence is measured in cuvettes after extraction into a suitable solvent. The two methods are compared as to their reproducibility and accuracy. Both methods are easy to perform and sensitive enough to determine dipyridamole in concentrations that would be expected in the plasma of patients being treated with this drug.

MATERIALS AND METHODS

Reagents

All reagents and solvents used were of guaranteed reagent grade (E. Merck, Darmstadt, G.F.R.). All solvents used in the TLC procedure were distilled prior to use.

Extraction solvent

(a) Fluoridensitometric method: A mixture of diethyl ether—dichloromethane (80:20) was used.

(b) Spectrofluorimetric method: A mixture of hexane—isoamyl alcohol (95:5) was used.

TLC developing solvent

A mixture consisting of ethyl acetate—methanol—28% ammonia (85:10:5) was used; this solvent was prepared immediately before use.

Dipping solution

Paraffin wax (m.p. 42—44°) was dissolved in light petroleum (b.p. 40—60°), 140 g in 2 l.

Stock solutions

Stock solutions of dipyridamole were prepared by weighing about 10 mg accurately on a Mettler ME22 electronic microbalance and adding sufficient distilled methanol to result in a solution containing 2 μg of dipyridamole per μl of methanol from which a solution containing 200 ng of dipyridamole per μl was prepared. These solutions were prepared fresh before each series of determinations.

Buffer solution

Aqueous solutions of 0.2 *M* Na_2CO_3 and 0.2 *M* $NaHCO_3$ were mixed until a pH of 10 was reached.

Apparatus

A Perkin-Elmer MPF3 spectrofluorimeter equipped with a Xenon lamp and a thin-layer scanning attachment was used to measure the fluorescence of the spots for the thin-layer procedure. The excitation wavelength used was 380 nm while a cut-off filter of 430 nm was selected for the emitted light. The instrument was used in the reflectance mode.

Fluorescence of dipyridamole when determined by means of the spectrofluorimetric method was measured on a Perkin-Elmer Model 204 spectrofluorimeter equipped with a xenon lamp. The excitation wavelength used was 405 nm and the emission wavelength 495 nm. The intensity of the fluorescence was measured by a disc integrator coupled to the recorder. The peak intensity was measured for a period of 20 sec and the number of counts recorded.

Thin-layer chromatographic equipment

Pre-coated silica gel TLC plates (Merck) with a layer thickness of 0.25 mm and without a fluorescence indicator were used. The extract was applied to the plate by means of a 5-μl micro capillary. The TLC plates were developed in a Shandon Chromatank (Shandon Scientific, London, Great Britain).

Analytical procedures

TLC—fluoridensitometric method. To 1 ml of plasma was added 1 ml of carbonate buffer (pH 10) and the mixture was extracted with 5 ml of distilled diethyl ether—dichloromethane (80:20) by shaking for 15 min on a mechanical shaker. The phases were separated by centrifugation (800 *g*, 2000 rpm, swing rotor), the organic phase transferred to a conical evaporation tube and dried at 50° under a stream of nitrogen. The residue was dissolved in 250 μl of redistilled dichloromethane and 5 μl were applied to the thin-layer plate. The plate was developed in the developing solvent to a height of 6 cm in an unsaturated atmosphere, dried with a stream of air at room temperature, left in the dark for 10 min and then dipped in the paraffin wax solution. After drying as described above, the plate was again left in a dark place for 10 min to allow stabilisation of the fluorescence, after which time it was ready for scanning.

Quantitation was done by processing five standard concentrations and spotting the concentrated extracts in duplicate on two different positions on the plate. Peak heights were measured, averaged, and plotted on a graph against concentration. The equation for this line, which conformed to a power curve, was derived through regression analysis and the concentrations of unknown samples were calculated by substituting their peak heights into this equation.

Spectrofluorimetric method. To 1 ml of plasma was added 1 ml of buffer (pH 10) and the mixture extracted with 8 ml of hexane—isoamyl alcohol (95:5) by shaking for 15 min on a mechanical shaker. After separation of the phases by centrifugation, 3 ml of the organic phase were transferred to a

quartz cuvette and the fluorescence measured using the conditions and equipment described above. Quantitation was done by measuring the intensity of fluorescence of five standards spanning the expected range of concentrations and plotting the number of counts vs. concentration. An equation for the best straight line fit through these points was obtained by linear regression analysis and the unknown concentrations were calculated by substitution of their peak heights into this equation.

Interference from other drugs

The drugs listed in Table I, in concentrations higher than would be expected in the therapeutic situation, together with dipyridamole (1 μg/ml), were subjected to the procedures described for both the thin-layer and spectrofluorimetric procedures. The fluorescence of the contaminated samples was measured and compared with an unadulterated sample. No interference was found.

TABLE I

DRUGS TESTED FOR INTERFERENCE

Acebutolol*	Hydrochlorothiazide
Amitryptiline	Lorazepam
Bamipine	Norephedrine
Benzoctamine	Penbutolol*
Carbamazepine	Procainamide*
Chlormezanone	Procaine*
Chlorpromazine*	Propranolol*
Clothiapine	Quinidine*
Diazepam	Salicylamide
Diphenhydramine	Thioridazine
Ephedrine	Trifluoroperazine
Flurazepam	Trimipramine

*Compounds with intrinsic fluorescence.

RESULTS

TLC—fluoridensitometric method

This method proved to be very good in the concentration range exceeding 50 ng/ml. It was found that the calibration curve of peak height vs. concen-

TABLE II

REPRODUCIBILITY OF THE TLC—FLUORIDENSITOMETRIC METHOD

Concentration spiked (ng/ml)	Peak height (mean ± S.D.)	*n*	Coefficient of variation
200	13.3 ± 1.2	3	9
400	31.0 ± 3.6	3	11
800	83.3 ± 9.6	3	12
1200	143.7 ± 5.9	3	4.1
1600	102.7 ± 9.6	3	4.8

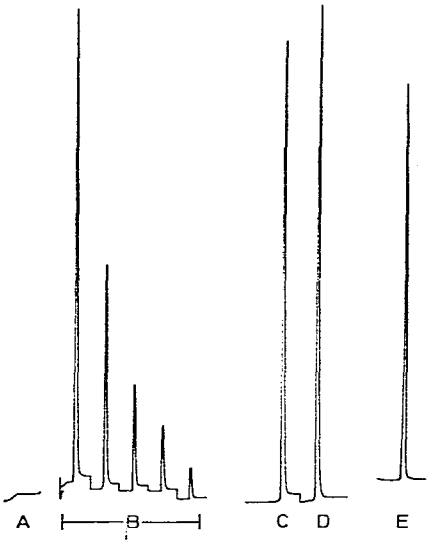

Fig. 2. Representative tracing of a TLC fluoridensitogram. (A) Plasma blank. (B) Standards from 400 ng/ml to 25 ng/ml. (C) 1200 ng dipyridamole per ml plasma extracted and compared with D, the expected amount. (E) Extract of a patient's plasma.

tration results in a line that fits a power curve very well (Table II). The reproducibility is also illustrated in Table II. A representative tracing of a TLC—fluoridensitogram of standards and a sample extract is illustrated in Fig. 2.

Spectrofluorimetric method

This method can be used for concentrations from 50 ng/ml upwards. The calibration graphs exhibit linear characteristics to a concentration of 1800 ng/ml. The particulars for the calibration graph as well as the reproducibility of the extraction procedure are illustrated in Table III.

TABLE III

REPRODUCIBILITY OF EXTRACTION OF SPECTROFLUORIMETRIC METHOD

Concentration spiked (ng/ml)	Counts (mean ± S.D.)	*n*	Coefficient of variation
100	21 ± 1	3	4.8
200	39 ± 2	3	5.1
400	75 ± 4	3	5.6
800	137 ± 4	3	3.1
1200	207 ± 10	3	4.7
1600	261 ± 4	3	1.4
1800	294 ± 5	3	1.7

COMPARISON OF SENSITIVITY OF THE TWO METHODS

The sensitivity limits of the two methods were compared by spiking serum with dipyridamole so as to obtain a range of concentrations from 10 ng/ml to 200 ng/ml. After extraction and evaporation of the extraction solvent 200 μl of dichloromethane were added to the residue and 10 μl portions of the different extracts were determined by means of the methods described above. In the case of the spectrofluorimetric method 10 μl of the reconstituted extract were added to 3 ml of hexane—isoamyl alcohol and the fluorescence was measured. When a comparison was made in this way the TLC—fluoridensitometric method proved to be more sensitive than the spectrofluorimetric method. It was found that as little as 15 ng/ml of dipyridamole would give a 20% of full-scale deflection when analyzed by means of the TLC—fluoridensitometric method, while giving virtually no response when using the spectrofluorimetric method. The minimum detectable quantity when using the latter method in the manner described above was 100 ng/ml.

EXTRACTION EFFICIENCY

To 1 ml of serum were added 1200 ng of dipyridamole, extracted with distilled diethyl ether—dichloromethane (80:20) and compared by means of the TLC—fluoridensitometric method with the expected theoretical amount. Extraction efficiency proved to be 92% (Fig. 2C and D). The extraction efficiency for the extraction used in the spectrofluorimetric method was 94%.

DISCUSSION

When the investigations into a method for determining dipyridamole were started it was found that certain solvents had a decomposing effect on the drug. It was found that in concentrations of 500 ng/ml the fluorescence disappeared when left overnight in chloroform and carbon tetrachloride, but remained unaffected when dissolved in dichloromethane, hexane, dichloromethane—diethyl ether (20:80), hexane—isoamyl alcohol (95:5) or methanol. In the light of these findings it was decided to prepare fresh standard solutions before each series of determinations. During the development of the TLC—fluoridensitometric method it was found that the quality of the plates had a marked effect on the fluorescence of the spots and that in some instances there was a significant difference in the intensity of the fluorescence of the same quantity of dipyridamole when applied on different positions on the plates. It was therefore necessary to spot each extract twice, at different positions on the plate. This, together with the observation that the calibration graph had to be constructed from five concentrations due to the non-linear characteristics thereof, had the effect that only five determinations could be done on one plate.

The effect of paraffin wax on the fluorescence of the spots [5] was quite dramatic and it was found that the intensity of dipyridamole fluorescence on the plate increased fifteen-fold. The mechanism of this effect is not clear but it may be due to a decrease of the surface scattering of the plate. The stability of the fluorescence of the spots was also investigated and it was found that

during the first 10 min after dipping of the plate there was a slight increase in the fluorescence but after this time the fluorescence remained stable for about 2 h, after which there was steady decline.

Since no internal standard is used, a crucial step in the procedure is the transfer of the organic phase, after initial extraction, to the evaporation tube. For the method to be accurate and reproducible it is essential that the amount of solvent transferred should be reproducible. Investigation of this procedure proved that the mean weight of solvent transferred was 4.4 g (5.2 ml) with a coefficient of variation of 2.7%, which is adequate for this kind of procedure.

With the number of samples that had to be assayed for the bioavailability study in mind it was decided to investigate the possibility of measuring the fluorescence of dipyridamole in cuvettes after a single extraction. This proved to be quite feasible and the method also proved to be amenable to a large number of samples since the procedure is very simple and the extracted dipyridamole was stable for several hours in the solvent employed.

To determine the correlation between the two procedures control samples containing two different concentrations of dipyridamole were made up and analysed without revealing the concentration to the analyst. Analyses were made on four different occasions and the results are displayed in Table IV. This result also serves to illustrate the accuracy and reproducibility of the two methods.

TABLE IV

DATA TO ILLUSTRATE THE PRECISION AND ACCURACY AND THE CORRELATION BETWEEN THE TLC—FLUORIDENSITOMETRIC AND SPECTROFLUORIMETRIC METHODS

Concentration spiked (ng/ml)	*n*	Amount recovered (ng/ml)	
		Spectrofluorimetric method (mean ± S.D.)	TLC method (mean ± S.D.)
270	4	259 ± 10.9	267.6 ± 14.3
680	4	665 ± 16.1	699.8 ± 28.7

TABLE V

CORRELATION BETWEEN SPECTROFLUORIMETRIC AND TLC—FLUORIDENSITOMETRIC METHODS WHEN A PATIENT'S PLASMA IS ANALYSED

Sample	Time after medication (h)	Concentration found (ng/ml)	
		Spectrofluorimetric method	TLC method
B1	1	1426, 1626	1380, 1503
B2	2	290, 320	316, 304
B3	4	154, 182	166, 177
B4	8	230, 270	251, 274
B5	12	106, 126	116, 150

A possible disadvantage of the spectrofluorimetric procedure is that it may be less selective than the TLC method due to the possible interference of metabolites. Since the extraction may not be selective it is possible that metabolites may contribute to the fluorescence and result in a falsely high concentration. To rule out this possibility a normal healthy volunteer was given 100 mg of dipyridamole. Blood was collected at 1, 2, 4, 8 and 12 h after medication. The samples were analysed by means of both the methods. The results, displayed in Table V, show a very good correlation between the two methods and it would appear as if metabolites do not significantly contribute to the value obtained by the spectrofluorimetric method when a patient receives a single dose of the drug.

The stability of dipyridamole in serum when stored at −20° was also determined; over a period of 22 days no deterioration could be found.

From the above it is therefore evident that the two methods are comparable in accuracy and precision but that the spectrofluorimetric method is more amenable to the analysis of a large number of samples.

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