Journal of Chromatography, 272 (1983) 157—165
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1484

DETERMINATION OF FLUPHENAZINE IN PLASMA BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

C.M. DAVIS* and D.C. FENIMORE*

Texas Research Institute of Mental Sciences, 1300 Moursund Avenue, Houston, TX 77030 (U.S.A.)

(First received April 13th, 1982; revised manuscript received August 26th, 1982)

SUMMARY

Determination of fluphenazine in blood plasma by in situ fluorescent detection after separation by high-performance thin-layer chromatography is described. Enhancement of fluorescent emission of the drug is accomplished by exposure to UV light in the presence of paraffin oil which permits a limit of detectability of approximately 0.1 ng/ml in blood plasma. Thirty samples or more can be processed in a 7-h period with excellent precision (less than 3% relative standard deviation at 2.5 ng/ml). Investigation of extraction procedures, chromatographic conditions, photodevelopment, and fluorescent detection are described.

INTRODUCTION

Fluphenazine (FPZ) is among the most potent of the phenothiazine drugs used in the treatment of schizophrenia. Esterification of the hydroxyl group in the piperazine side chain with heptanoic or decanoic acid yields dosage forms for intramuscular injection which release the drug slowly over a period of 1 to 4 weeks thus simplifying maintenance of therapeutic levels in the blood and central nervous system. The concentration of FPZ circulating under these conditions is quite low and has been shown by radiolabeled experiments to be less than 1 ng/ml of blood plasma over most of the dosage period [1]. Determination of drug blood levels at such low concentrations under the constraints of limited sample volume, minimal assay time, and acceptable cost of analysis, pose serious problems particularly where large patient populations are involved in therapeutic drug monitoring programs.

Procedures for the determination of FPZ reported in recent years are based primarily on gas chromatography with electron-capture detection [2, 3] or

*Present address: Clarke Analytical Systems, P.O. Box 744, Sierra Madre, CA 91024, U.S.A.

0378-4347/83/0000—0000/$03.00

with nitrogen specific detection [4, 5]. The sensitivity of these methods are, for the most part, inadequate for blood level monitoring under therapeutic regimens with perhaps the exception of the procedure described by Dekirmenjian et al. [5] which employs the acetylated derivative of FPZ with a reported subnanogram limit of detectability. A radioimmunoassay reported by Wiles and Franklin [6] achieved very high sensitivity but cross-reacted significantly with some of the metabolic products of FPZ, as well as, with other phenothiazine drugs.

This report describes a sensitive and selective procedure involving in situ fluorescent detection of FPZ after separation by high-performance thin-layer chromatography (HPTLC). The improvements in TLC methodology and materials which have resulted in HPTLC permit sensitive, selective, and rapid assays [7, 8] that may be applied economically to large numbers of samples. The limit of detectability for FPZ by the present procedure is approximately 0.1 ng/ml of plasma using a 4-ml sample. Thirty to sixty patient samples and calibration standards (depending on HPTLC plate size) can be processed in less than 6 h with a precision of better than 3% relative standard deviation.

EXPERIMENTAL

Materials and apparatus

Hydrochloride salts of FPZ and trifluopromazine (TFP) were obtained from E.R. Squibb & Sons (New Brunswick, NJ, U.S.A.) and the Upjohn Co. (Kalamazoo, MI, U.S.A.), respectively. Toluene, heptane, isoamyl alcohol, and acetone (Fisher Scientific, Pittsburg, PA, U.S.A.) were distilled in an all-glass system and stored in glass bottles with PTFE-sleeved glass stoppers. Hydrochloric acid, ammonium hydroxide, sodium carbonate and paraffin oil were also purchased from Fisher Scientific. Absolute ethanol (U.S. Industrial Chemical Co., Tuscola, IL, U.S.A.) was used as received. HPTLC plates (Silica Gel 60, Cat. No. 5633, E. Merck, Darmstadt, G.F.R.) were pre-cleaned by overnight development in absolute ethanol in the presence of vapor from concentrated ammonium hydroxide.

Either a Zeiss KM-3 (Carl Zeiss, Oberkochen, G.F.R.) or a Shimadzu CS-910 (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.) scanning densitometer equipped with a mercury lamp was used for fluorodensitometric determination, and samples were applied to the HPTLC plates with a Contact Spotter, Model 1010 (Clarke Analytical Systems, Sierra Madre, CA, U.S.A.). All glassware was silylated by a vapor phase method [9].

Stock standards

A standard containing 250 ng/ml and 300 ng/ml FPZ and TFP respectively was prepared in heptane containing 25% ethanol and 0.05% *n*-dodecane. The amount of FPZ and TFP in 40 μl of this standard was equivalent to the central point (2.5 ng/ml) of the calibration curve produced by extracts of plasma standards and as such served as an external standard to monitor the assay procedure. A standard containing 120 ng/ml TFP was prepared in heptane to introduce 12 ng of the internal standard to the plasma before extraction. An

additional standard containing 10 μg/ml of FPZ in heptane was prepared for the standard curve. All weights of FPZ and TFP represent the free form of the drug.

Standard curve

Standard curves were prepared by evaporating 125 μl of the 10 μg/ml FPZ standard under nitrogen at 40°C in a 250-ml volumetric flask. The drug was redissolved in 100 μl of 0.05 *N* hydrochloric acid, diluted to volume with drug-free plasma, and mixed for approximately 30 min. Additional dilutions were obtained by transferring 50-ml and 10-ml aliquots of the 5 ng/ml plasma standard to 100-ml volumetric flasks and adding drug-free plasma to volume. These standards were then transferred in 4-ml amounts to 30-ml silylated screw-top test tubes fitted with PTFE-lined tops and frozen at −70°C for further use.

Procedure

The following solutions were pipetted into 30-ml silylated screw-top test tubes containing 4 ml of patient plasma or plasma standard: 100 μl of internal standard solution, 0.6 ml of saturated sodium carbonate solution, and 20 ml distilled heptane containing 0.50% isoamyl alcohol. The tubes were capped with PTFE-lined closures, mixed for 45 min on a tube rocker (Lab-Industries, Berkeley, CA, U.S.A.) and centrifuged at 1800 *g* for 30 min. The aqueous phase was then frozen by immersing each tube momentarily in dry ice—acetone, and the organic phase was decanted into another silylated screw-top test tube containing 2 ml of 0.05 *N* hydrochloric acid. The tubes were then shaken for 15 min, centrifuged at 1500 *g* for 5 min, and the organic layer was removed by aspiration and discarded. The samples were then back-extracted by the addition of 0.3 ml saturated sodium carbonate solution and 4.5 ml of distilled pentane followed by shaking for 15 min and centrifugation at 1500 *g*. The aqueous phase was again frozen and the organic layers decanted into Reacti-vials (Pierce, Rockford, IL, U.S.A.). The pentane was removed by evaporation under nitrogen at 40°C, and the residue was redissolved in 50 μl heptane—ethanol—*n*-dodecane (75:25:0.05). Each extracted solution was then deposited in the separate indentations of the Contact Spotter as was a 40-μl volume of the spotting standard. After evaporation at the "low" temperature setting of the Contact Spotter, the residues were transferred to a 10 × 10 cm pre-washed HPTLC plate, 8 mm from the edge of the plate.

The plates were developed in a pre-equilibrated linear development chamber (Camag, Applied Analytical Industries, Wilmington, NC, U.S.A.) containing toluene—acetone (60:40). This solvent moved the FPZ and internal standard to an R_F of 0.1, and interfering plasma components moved with the solvent front. A second, pre-equilibrated development in toluene—acetone—ammonium hydroxide (60:40:2) was carried out to a distance 4 cm from the origin and moved the FPZ and internal standard to R_F values of 0.2 and 0.5, respectively. Chromatographic separation occurred in an air-conditioned laboratory at approximately 60% relative humidity and 23°C.

After evaporation of solvent the plate was placed in 5% paraffin oil (Saybolt viscosity 125/135) in toluene for 15 min. The toluene was evaporated

from the adsorbent layer, and the plate was exposed to UV light (Blak-Ray B100A, Ultraviolet Products, San Gabriel, CA, U.S.A.) for 15 to 20 min. The photodeveloped plate was then scanned in the fluorescent mode at 254-nm excitation wavelength with a 400-nm cut-off filter.

RESULTS AND DISCUSSION

The phenothiazine drugs can be detected on TLC plates by a variety of reagent sprays [10] or by UV absorption with or without preliminary reagent treatment [11, 12]. The sensitivity achieved with these methods, however, is not sufficiently sensitive to permit reliable quantitation of FPZ at the plasma concentration associated with therapeutic dosage. Fluphenazine, like many of the phenothiazine drugs, is fluorescent under appropriate conditions, a quality that would appear to offer some advantage with respect to sensitivity and selectivity of detection. Unfortunately, the fluorescent yield of FPZ adsorbed on silica gel is not adequate for determination when chromatographed plasma extracts are examined by conventional fluorescent scanning techniques.

In attempts to enhance the fluorescence of FPZ, the adsorbent layer of the HPTLC plate was exposed to paraffin oil, a treatment which had been shown to increase fluorescent yield when applied to other classes of compounds [13]. This recourse was not successful in itself, but it was observed that when the paraffin oil-treated plate was exposed to UV light, small amounts of FPZ became visible after 3 or 4 min and reached maximum emitted intensity after 15—20 min. If the plate was exposed for periods longer than 30 min, background fluorescence increased thus diminishing the overall sensitivity. Photodevelopment in the absence of paraffin oil did not enhance fluorescence.

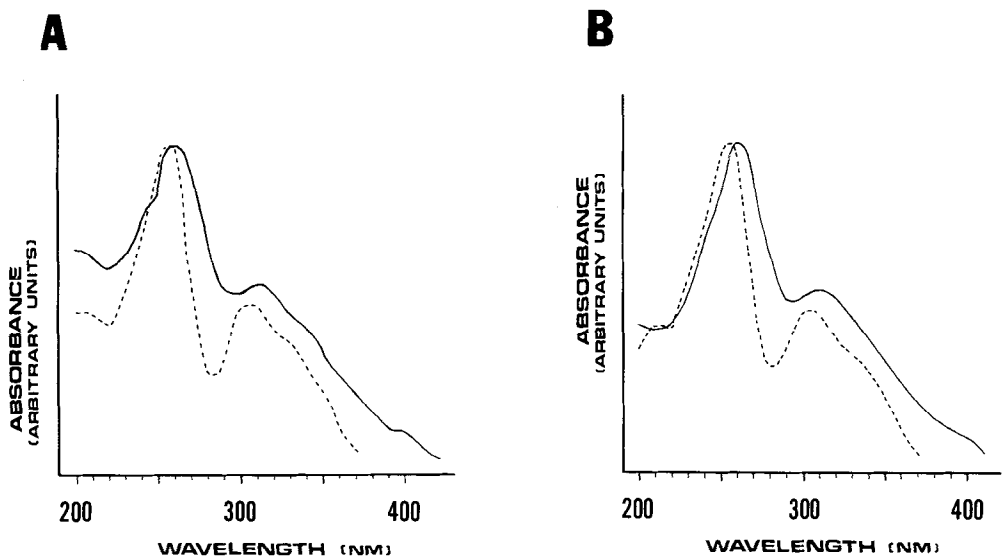

Fig. 1. (A) UV spectrum of fluphenazine (FPZ) before (— — —) and after (——) photodevelopment procedure. (B) UV spectrum of trifluprómazine (TFP), internal standard, before (— — —) and after (——) photodevelopment procedure.

The fluorescent products of both FPZ and the internal standard, TFP, formed in this manner were stable on the HPTLC plates for periods as long as 6 months. Although no attempts were made to characterize these products, their UV spectra were different from the parent compounds (Fig. 1). The fluorescent product of FPZ was examined on an Aminco Bowman Spectrofluorometer equipped with a xenon lamp and was found to have major excitation wavelengths at 285 and 355 nm with emission maximum at 510 nm. In order to utilize one of these excitation maxima, it was originally elected to employ a xenon lamp attachment with the Shimadzu CS-910 densitometer; but sensitivity and stability were difficult to maintain with this configuration. Consequently, the less expensive mercury lamp attachment was evaluated at the various mercury emission lines, and it was found that maximum excitation occurred at the 253.7-nm line resulting in a 17-fold increase in response over the best fluorescent emission obtained with the xenon lamp.

The extraction of drug and internal standard from plasma followed a fairly straightforward scheme of liquid—liquid partitioning with back-extractions at appropriate conditions of acidity and basicity. It was found that certain precautions had to be taken, however, to exclude fluorescent contamination from reagents. The sodium carbonate used to prepare the saturated pH 11.6 solution contained an unacceptably large amount of fluorescent material and the 0.05 *N* hydrochloric acid showed trace contamination. These contaminants were removed by multiple extractions with 30% volumes of distilled heptane. Once cleaned, the solutions were held in glass bottles sealed with PTFE-sleeved glass stoppers. When contamination was suspected, it was readily checked by processing a water blank through the entire procedure.

Plasma extracts contained fluorescent substances that emitted at the same wavelength range as FPZ, but most of this interference was removed by careful selection of pH and by the use of non-polar extraction solvents. Fig. 2 demonstrates the extraction of FPZ into pentane from plasma adjusted to specific pH values. The addition of 0.6 ml saturated sodium carbonate solution to 4 ml plasma as described in the procedure resulted in a pH of 10 which was sufficient to affect maximum partitioning without co-extracting interfering substances.

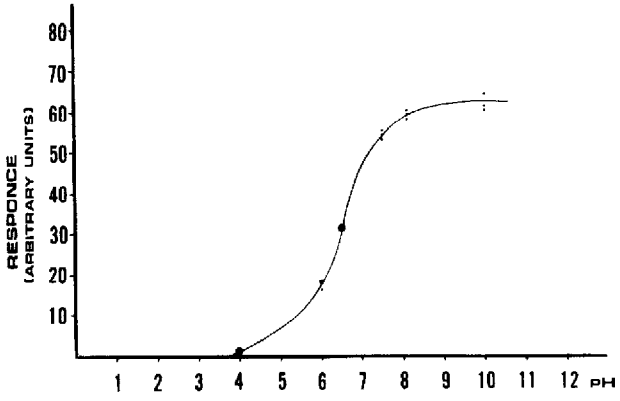

Fig. 2. Effect of pH on the partitioning of fluphenazine (FPZ) out of plasma. Spectrophotometric measurements were carried out on the FPZ which extracted into pentane.

A number of organic solvents were evaluated with respect to extraction efficiency. The more polar solvents such as diethyl ether and ethyl acetate extracted interfering substances while non-polar solvents such as pentane or heptane provided clean extracts with fairly good recovery. The addition of a small amount of isoamyl alcohol (0.50%) to the heptane resulted in a lower coefficient of variation when the same plasma sample was extracted a number of times. Larger amounts of isoamyl alcohol added to the heptane increased the extraction of interfering substances from the plasma. The complete extraction procedure as described resulted in approximately 75% recovery of drug, however, about 95% of the drug was recovered during the back extraction steps suggesting the greatest loss occurred in the initial extraction of plasma.

TABLE I

R_F VALUES OF 32 BASIC DRUGS SCREENED FOR INTERFERENCE

The solvent system used was toluene—acetone—ammonia (60:40:1).

Drugs	R_F	Relative in situ fluorescent yield
Mesoridazine	0.00	6
Protriptyline	0.06	7
Desipramine	0.08	0
Thiothixine	0.09	14
Acetophenazine	0.09	60
Carphenazine	0.11	70
Nortriptyline	0.14	0
Methdilazine	0.15	0
Perphenazine	0.15	2
Butaperazine	0.19	64
Fluphenazine	0.20	40
Prochlorperazine	0.23	3
Trifluoperazine	0.23	47
Clozapine	0.27	0
Nicotine	0.28	0
Promazine	0.30	1
Caffeine	0.32	0
Imipramine	0.35	1
Chlordiazepoxide	0.35	10
Ethopropazine	0.36	1
Thioridazine	0.37	16
Doxepin	0.38	2
Promethazine	0.41	1
Chlorpromazine	0.43	7
Loxapine	0.43	0
Haloperidol	0.43	0
Amitriptyline	0.45	0
Flurazapam	0.47	0
Triflupromazine	0.47	48
Chlorprothixine	0.57	100
Methotrimeprazine	0.64	1
Diazepam	0.73	0

Samples were mixed with extraction solvent by gently rocking in order to avoid excessive emulsification. Centrifugation at 1800 *g* for 30 min yielded a clean separation without a pronounced emulsion layer between the two phases. This emulsion layer must be minimized to maintain good recovery.

After the extracts were taken to dryness, they were redissolved in 50 μl of a solvent mixture containing 0.05% *n*-dodecane. The 25 nl of *n*-dodecane which remained with the extract after evaporation on the Contact Spotter were necessary to insure complete sample transfer to the HPTLC plate during the sample application process. Simultaneous transfers of 15 extracts were placed 6 cm apart on each of two opposing edges of a 10 × 10 cm plate for separation of 30 samples with each chromatographic development. The Camag linear development chamber applied developing solvent to these opposing edges, and sample separation occurred as the solvent fronts moved towards

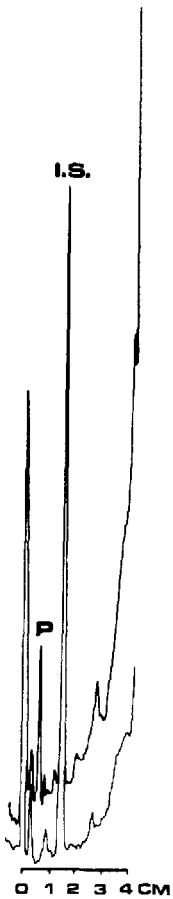

Fig. 3. Fluorescent scan of a patient on Prolixin (fluphenazine) therapy at a concentration of 0.6 ng/ml. Lower trace is blank plasma containing the internal standard. Peaks: P = FPZ; I.S. = TFP, internal standard.

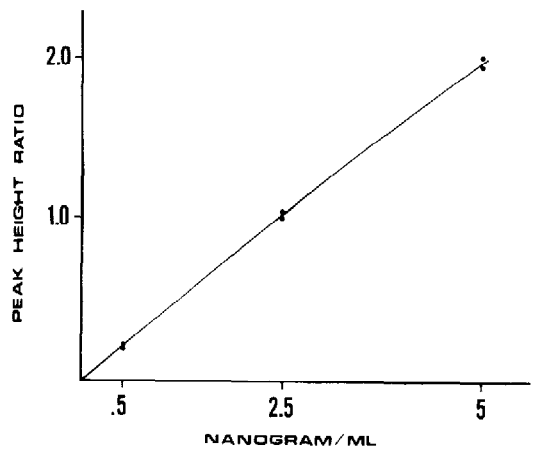

Fig. 4. Standard curve of the peak height ratio of FPZ/I.S. extracted from plasma. Each concentration was assayed in duplicate.

one another at the center of the plate, thus chromatographing the 30 samples in a single 7-min period. Pre-equilibration of the HPTLC plate for 2 min with vapors of the developing solvent was necessary to reduce "edge effect" and provide more uniform R_F values among sample lanes. The relative standard deviation obtained from the repeated analysis (n = 12) of the 2.5 ng/ml plasma standard using a linear development chamber (2.8%) was approximately half the relative standard deviation found with the conventional chromatography tank (5%).

Table I shows the R_F values and relative fluorescent responses for 32 drugs screened for possible interference. The R_F values presented here are slightly lower than those obtained by the dual development system, because the drugs were separated by only the final solvent system. Since most of these basic drugs have very little, if any, fluorescent emission at an excitation wavelength of 253.7 nm, they do not contribute to interferences in the detection process. A chromatographic tracing of a plasma extract after development in both solvent systems is shown in Fig. 3 and represents 0.6 ng/ml of FPZ from a patient on Prolixin therapy.

TABLE II

PLASMA FLUPHENAZINE LEVELS

Patient No.	Days of FPZ treatment	Dosage of FPZ*	Interval** (days)	Plasma FPZ (ng/ml)
I	1	a	1	1.06
	20	a	1	0.72 ± 0.02
	45	a	4	0.60 ± 0.05
	49	a	1	0.70 ± 0.05
	87	a	5	0.45 ± 0.05
	104	a	9	0.55
	118	a	10	0.53 ± 0.03
	125	a	3	0.25 ± 0.05
	133	a	9	0.35 ± 0.05
	140	a	2	0.35 ± 0.05
II	1	a	1	0.38 ± 0.08
	14	a	1	0.23 ± 0.08
	28	a	1	0.35 ± 0.05
	38	a	8	0.32
III	1	c	0.5	0.56
	7	a	4	ND***
	14	a	10	ND
	31	a	14	0.23
IV	1	c	0.5	1.35 ± 0.05
	18	a and b	3 0.5	2.5
V	1	b	0.5	0.65
	7	a	4	0.30
VI	1	c	0.5	1.40 ± 0.05

*a = Fluphenazine decanoate, 25 mg every 2 weeks; b = fluophenazine·HCl, 5 mg three times per day; c = fluphenazine·HCl, 10 mg three times per day.
**Interval between dosage and blood sampling.
***ND = not detected.

The capability of HPTLC for accepting many samples simultaneously greatly simplifies calibration, for little additional effort is required to include several standards with any particular determination. Standard curves, such as that illustrated in Fig. 4, prepared from duplicate plasma extracts over three concentrations were run concurrently with each group of patient samples. This practice effectively excluded those errors which may arise from small variations in chromatographic conditions in processing each discrete HPTLC plate. Analysis of plasma containing 0.5 ng/ml or 2.5 ng/ml of FPZ resulted in a relative standard deviation of 8.1% and 2.8%, respectively (n = 12).

The applicability of this method to the monitoring of patient blood levels during therapy with long-acting dosage forms of FPZ is shown in Table II. The values obtained were generally consistent with the amount and dosage form of the drug over extended periods of observation.

REFERENCES

1 S.H. Curry, R. Whelpton, P.J. DeSchepper, S. Vranckx and A.A. Schiff, Lancet, i (1978) 1217—1218.
2 L. Rivera-Calimlim and A. Siracusa, Commun. Psychopharmacol., 1 (1977) 233—242.
3 N.-E. Larsen and J. Naestoft, Med. Lab. Technol., 30 (1973) 129—132.
4 M. Franklin, D.H. Wiles and D.J. Harvey, Clin. Chem., 24 (1978) 41—44.
5 H. Dekirmenjian, J.I. Javaid, B. Duslak and J.M. Davis, J. Chromatogr., 160 (1978) 291—296.
6 D.H. Wiles and M. Franklin, Brit. J. Clin. Pharmacol., 5 (1978) 265—268.
7 W. Bertsch, S. Hara, R.E. Kaiser and A. Zlatkis, Instrumental HPTLC, Proceedings of the First International Symposium on Instrumentalized High Performance Thin-Layer Chromatography (HPTLC), Bad Dürkheim, West Germany, Dr. Alfred Hüthig Verlag, New York, 1980.
8 D.C. Fenimore and C.M. Davis, Anal. Chem., 53 (1981) 252A—266A.
9 D.C. Fenimore, C.M. Davis, J.H. Whitford and C.A. Harrington, Anal. Chem., 48 (1976) 2289—2290.
10 J.G. Kirchner, in A. Weissberger (Editor), Thin-Layer Chromatography, Techniques of Chemistry, John Wiley & Sons, New York, 2nd ed., 1978.
11 D.C. Fenimore, C.J. Meyer, C.M. Davis, F. Hsu and A. Zlatkis, J. Chromatogr., 142 (1977) 399—409.
12 D.C. Fenimore, C.M. Davis and C.J. Meyer, Clin. Chem., 24 (1978) 1386—1392.
13 R.S. Day, R.E. De Salamanca and L. Eales, Clin. Chim. Acta, 89 (1978) 25—33.