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Note

High-performance liquid chromatographic analysis for fluorescein and fluorescein monoglucuronide in plasma

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Fluorescein (F) is widely used as a diagnostic aid in medicine, especially in the practice of ophthalmology [1, 2]. Intravenous (i.v.) injection of F for fundus angiography has become an important diagnostic and research tool and has provided an understanding and classification of various diseases of the fundus [3, 4].

Although F has been determined in physiological fluids by measuring its absorbance [5], interference from extraneous absorbing substances has been reduced by the use of its fluorescent properties [6]. Previous investigations with F have assumed that the total fluorescence observed in plasma samples is due entirely to F. However recent studies have suggested that F is quickly converted to the monoglucuronide [7—9]. The fluorescence of the fluorescein monoglucuronide is lower than that of fluorescein, with the fluorescence ratio of the two dependent on pH and the combination of excitation and emission wavelengths [8, 10]. Previous studies have measured free F by the fluorometric determination of native sample, and the conjugate as F after the hydrolysis with either sodium hydroxide or β-glucuronidase [8, 11].

High-performance liquid chromatography (HPLC) with fluorescence detection described in this report provides a fast and sensitive determination of F and its major metabolite FG directly from plasma. In addition, this technique provides a level of sensitivity approaching that of gas chromatography but without the attendant difficulties.

EXPERIMENTAL

Reagents

The sodium salt of fluorescein was purchased from Sigma (St. Louis, MO,

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U.S.A.). Fluorescein monoglucuronide was prepared by Dr. Thomas Guenthner, Department of Pharmacology, University of Illinois Medical Center. Spectrograde methanol and PIC reagent A were supplied by Waters Assoc. (Milford, MA, U.S.A.). All solutions were prepared by using double-distilled water.

Subject and medication

One adult male volunteer was given 14 mg/kg fluorescein by bolus intravenous administration in the vein of the right arm. Venous blood samples were withdrawn from the vein of the left arm at 0, 1, 60, 120 and 240 min following drug administration. The samples were immediately centrifuged and the plasma kept frozen until analysis.

Chromatography

HPLC analyses were conducted using a Waters Assoc. Model 6000A pump coupled with a Waters Model U6K injection system. Separation was performed by ion-pair chromatography using a Waters Assoc. C_{18} bonded reversed-phase column (15 cm × 3.9 mm) with 5 μm particle size. The mobile phase consisted of 47% methanol in a 0.005 *M* aqueous solution of PIC reagent A (tetrabutylammonium phosphate) and was degassed at reduced pressure before use. The chromatographic system was operated at ambient temperature with an eluent flow-rate of 1 ml/min.

Fluorescence was measured with a Waters Model 420 fluorescence detector, using a 500-nm long-pass emission filter and a 450-nm band-pass excitation filter in conjunction with the F4T5/D fluorescence lamp.

Ultrafiltration

Plasma ultrafiltrates were prepared using the Amicon (Danvers, MA, U.S.A.) micropartition system with the YMT membrane. Serum samples (0.5 ml) were centrifuged at 2000 *g* for 20 min through the system [12]. Approximately 100 μl of each ultrafiltrate was injected onto the column.

RESULTS AND DISCUSSION

Method development

Ultrafiltration by centrifugation with the micropartition system is a simple, highly reproducible technique ideally suited for the determination of the free fraction of drugs in plasma [13]. In order to investigate the possible binding of F and FG to the YMT filter, standards were prepared in distilled water and centrifuged for 20 min at 2000 *g* through the filter. The YMT filter showed no significant binding of either F or FG (Table I).

Calibration curves were prepared from a series of six triplicate F and FG standards in water, ranging from 12.5 to 500 ng/ml. The samples were processed and the ultrafiltrate was measured using the chromatographic solvent system described previously. Linear regression analyses of peak heights versus the concentration of compounds of interest yielded linear calibration curves with $r^2 \geqslant 0.99$. The detection limit of the assay is approximately 10 ng/ml for both compounds of interest. A listing of these data is provided in Table II.

TABLE I

FLUORESCEIN AND FLUORESCEIN MONOGLUCURONIDE BINDING TO YMT FILTER

Samples were assayed in triplicate. Measured values are means ± standard error.

Drug	Actual (ng/ml)	Measured before filtration (ng/ml)	Measured in ultrafiltrate (ng/ml)
Fluorescein	100	99.6 ± 3.3	103.6 ± 0.3
Fluorescein monoglucuronide	100	102.4 ± 2.3	99.2 ± 3.6

TABLE II

CALIBRATION CURVE PARAMETERS

Linear regression analyses of the given data sets were carried out with y value being peak height and x value being concentration (F, FG); n samples (including duplicate or triplicate determinations) were utilized for each calibration curve with r^2 (correlation coefficient) being a measure of linearity, b representing the y intercept, m indicating the slope of the regression and C.V. the mean coefficient of variation.

Medium	Compound	Range (ng/ml)	n	b	m	r^2	C.V.
Water	F	12.5—500	19	0.018	0.001	0.99	0.1
Water	FG	12.5—500	19	0.005	0.001	0.99	0.6

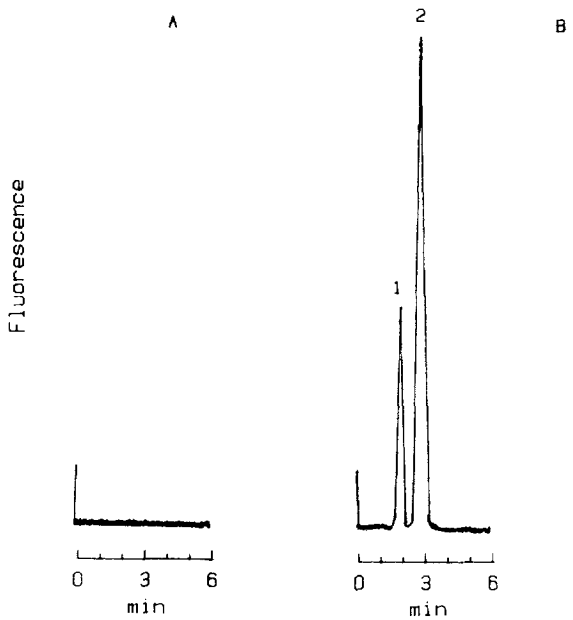

Fig. 1. HPLC chromatograms of (A) blank plasma ultrafiltrate; and (B) ultrafiltrate from a plasma sample containing 100 ng/ml FG (1) and 100 ng/ml F (2).

A calibration curve of F in control plasma was prepared in the concentration range 40—200 ng/ml and the ultrafiltrate analyzed. Linear regression analysis of peak height versus concentration of F in plasma yielded a linear calibration curve with a correlation coefficient $\geqslant 0.99$ and a slope of 0.001 ml/ng. Based on the concentration of free F no significant difference was observed in the chromatograms for standards prepared in water and standards prepared in plasma. The day-to-day precision of the assay based on variation in peak height was less than 3%.

A given chromatogram (Fig. 1A) of plasma ultrafiltrate without added F or FG yielded no peaks over the first 6 min under the chromatographic conditions described earlier. The chromatogram (Fig. 1B) of plasma ultrafiltrate from plasma previously supplemented with an equal concentration (100 ng/ml) of F and FG yielded both peaks of interest. The chromatograms of plasma ultrafiltrate from patients receiving fluorescein appeared to be identical to those of spiked plasma.

Protein binding

Adverse reactions to intravenous fluorescein used in fundus angiography are usually of minimum consequence. However, serious side-effects including anaphylactic reactions, acute pulmonary edema, respiratory obstruction and arrest, and hypotension with a shocklike reaction have been reported [14—16]. It has recently been suggested that the previously reported adverse effects of intravenously injected F may be related to the amount of protein binding of fluorescein [17]. For this reason an investigation of the interaction between human plasma and F and its primary metabolite FG using the micropartition system was performed.

Fluorescein is bound in the blood mainly to protein, particularly to albumin and erythrocytes [18, 19]. Previous investigations have reported the percent F binding to plasma proteins in the range 40—90%, while one recent paper has reported finding no significant protein binding of F using polyacrylamide gel electrophoresis [20].

Protein binding of F to the plasma proteins was investigated at three different F concentrations: 40, 80 and 100 ng/ml (Table III). Plasma was supplemented with F and an aliquot was processed by ultrafiltration. At all

TABLE III

PROTEIN BINDING OF FLUORESCEIN AND FLUORESCEIN MONOGLUCURONIDE

Blank plasma samples were supplemented with F or FG. Values reported are the mean of three separate determinations.

Drug	Actual concentration (ng/ml)	Concentration in ultrafiltrate (ng/ml)	Percentage bound
Fluorescein	40	7.8	80.5
	80	13.4	83.3
	100	15.2	84.8
Fluorescein monoglucuronide	100	57.4	42.6

TABLE IV

EFFECT OF FREEZING ON PLASMA PROTEIN BINDING OF FLUORESCEIN

Blank plasma samples were supplemented with F. Values reported are the mean three to four determinations.

Serum concentration (ng/ml)	Fresh ultrafiltrate concentration (ng/ml, ± S.E.)	Percentage bound	Frozen ultrafiltrate concentration (ng/ml, ± S.E.)	Percentage bound
40	7.8 ± 0.1	80.4	7.7 ± 0.6	80.6
80	13.5 ± 0.1	83.0	13.8 ± 0.5	82.7
100	15.3 ± 0.2	84.5	15.9 ± 0.6	84.1
200	31.2 ± 3.7	84.4	30.3 ± 0.2	84.8

three concentrations F was found to be protein-bound with the average binding calculated to be 82.9%. This corresponds to previous reports of 82.5% and 86.2% plasma protein binding of F [9, 21]. The protein binding of FG at a concentration of 100 ng/ml was found to be 42.6%, approximately one-half that found for an equivalent concentration of F (Table III).

The effect of freezing on protein binding of F was investigated at four different F concentrations (Table IV). Plasma with added F was divided in half, with half kept at room temperature and half frozen overnight. The following day protein binding of F was assessed. No significant change in protein binding was evident between the frozen and non-frozen samples at any of the concentrations used.

Clinical sample

Initial studies were performed on plasma samples from a male volunteer receiving 14 mg/kg F intravenously. Blood samples were withdrawn 0, 1, 60, 120 and 240 min after F administration and assayed as previously described. Detectable concentrations of F and FG were found in plasma ultrafiltrate at the stated times and are presented in Table V. It was observed that free F was

TABLE V

PLASMA ULTRAFILTRATE CONCENTRATIONS OF FLUORESCEIN AND FLUORESCEIN MONOGLUCURONIDE AFTER i.v. ADMINISTRATION OF FLUORESCEIN

Fluorescein (14 mg/kg) was administered by bolus intravenous injection in the right arm. Venous blood samples were removed from the left arm at the stated times. The concentrations reported are the mean of three to four determinations.

Time (min)	Concentration (ng/ml)	
	F	FG
0	0	0
1	35,635	0
60	2661	37,000
120	727	23,982
240	156	14,952

rapidly converted to its glucuronide. At 1 min after i.v. administration free F was at its maximum concentration while there was no detectable free glucuronide. By 1 h there was approximately a fourteen fold increase in free metabolite over drug. This rapid metabolism of F to FG agrees with previously reported work [9].

The present paper describes an assay procedure for the rapid determination of F and its primary metabolite FG in plasma. The described assay is currently being used in this laboratory for clinical study of F and FG pharmacokinetics.

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REFERENCES

1 H.R. Novotny and D.L. Alvis, Circulation, 24 (1961) 82.
2 G.J. Menaker and M.L. Parker, Surgery, 27 (1950) 41.
3 J.D. Gass, Stereoscopic Atlas of Macular Disease — A Funduscopic and Angiographic Presentation, Mosby, St. Louis, MO, 1970, p. 29.
4 J.D. Gass, Differential Diagnosis of Intraocular Tumors — A Stereoscopic Presentation, Mosby, St. Louis, MO, 1974.
5 J.M. Crismon and F.A. Fuhrman, J. Clin. Invest., 26 (1947) 259.
6 G.J. Menaker and M.L. Parker, Surgery, 27 (1950) 44.
7 D. Dhumeaux, P. Berthelot, R. Fauvert and A. Preaux, Biol. Gastro-Enterol., 1 (1968) 37.
8 S.C. Chen, H. Nakamura and Z. Tamura, Chem. Pharm. Bull., 28 (1980) 2812.
9 M. Araie, M. Sawa, S. Nagataki and S. Mishima, Jap. J. Ophthalmol., 24 (1980) 346.
10 S.C. Chen, H. Nakamura and Z. Tamura, Chem. Pharm. Bull., 28 (1980) 1403.
11 J.M. Webb, M. Fonda and E.A. Brouwer, J. Pharmacol. Exp. Ther., 137 (1961) 141.
12 J. Thompson, A. Moschella, W.F. Bowers, W. MacMahon and D. Sgoutas, Clin. Chem., 27 (1981) 1057.
13 J.A. Sophianopoulos, S.J. Durham, A.J. Sophianopoulos, H.C. Ragsdale and W.P. Cropper, Arch. Biochem. Biophys., 187 (1978) 132.
14 S. Ingelstedt and B. Ivstam, Int. Arch. Allergy Appl. Immunol., 1 (1950) 157.
15 J.B. Hess and R.I. Pacurariu, Amer. J. Ophthalmol., 82 (1976) 567.
16 M.R. Stein and C.W. Parker, Amer. J. Ophthalmol., 72 (1971) 861.
17 C.M. Arroyaue, R. Wolbers and P.P. Ellis, Amer. J. Ophthalmol., 87 (1979) 474.
18 L. Anderson, A. Rehnstroem and D.L. Eaker, Eur. J. Biochem., 20 (1971) 371.
19 J.M. Crimson and F.A. Fuhrman, J. Clin. Invest., 26 (1947) 259.
20 D.C. Ianacone, N.T. Felberg and J.L. Federman, Arch. Ophthalmol., 98 (1980) 1643.
21 M. Affrime and M. Reidenberg, Eur. J. Clin. Pharmacol., 8 (1975) 267.