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DETERMINATION OF THE ANTIBIOTIC FLUDALANINE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A PACKED-BED, POST-COLUMN REACTOR WITH *o*-PHTHALALDEHYDE AND 2-MERCAPTOETHANOL

D.G. MUSSON*, S.M. MAGLIETTO and W.F. BAYNE

Merck Sharp & Dohme Research Laboratories, West Point, PA 19486 (U.S.A.)

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SUMMARY

Fludalanine is a novel anti-bacterial agent active against gram-negative and gram-positive bacteria. A high-performance liquid chromatographic assay has been developed using ion-pair chromatography to resolve fludalanine and the internal standard 3,3-difluoro-D-alanine from plasma and urine background. The mobile phase contains sodium dodecyl sulfonate and methanol in a phosphate buffer. Fludalanine is derivatized post-column with *o*-phthalaldehyde via a packed-bed chemical reactor. The adduct is detected fluorometrically. The plasma and urine assays are sensitive to 0.25 and 0.5 μg/ml, respectively.

INTRODUCTION

Fludalanine, 2-deutero-3-fluoro-D-alanine (I), is a broad spectrum antibiotic [1]. The antibacterial action results from a sequential blockade of the biosynthesis and incorporation of D-alanine into the bacterial cell wall [2]. The metabolism of fludalanine in animals (Fig. 1) involves its initial oxidation to 3-fluoropyruvate (II); this metabolite is then reduced to 3-fluoro-L-lactate (III) [3]. In addition, inorganic fluoride is an end metabolite [4].

Fludalanine is administered with pentizidone, D-4-(4-oxo-2-pentenc-2-amino)-3-isoxazolidinone (IV), a prodrug of cycloserine. The combination potentiates the individual antimicrobial activity of fludalanine and of cycloserine. The combination also eliminates the self-reversal phenomenon of fludalanine [1, 4].

A chemical assay for the quantification of fludalanine in plasma and urine has not been available. Generally, the detection of an amino acid by high-performance liquid chromatography (HPLC) involves either pre- or post-

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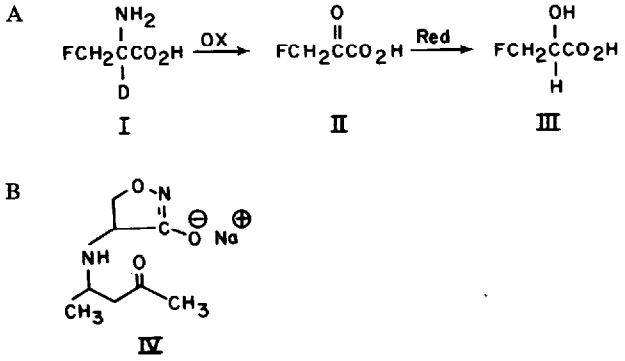

Fig. 1. (A) Metabolism of fludalanine in animals. (B) Structural formula of pentizidone (IV).

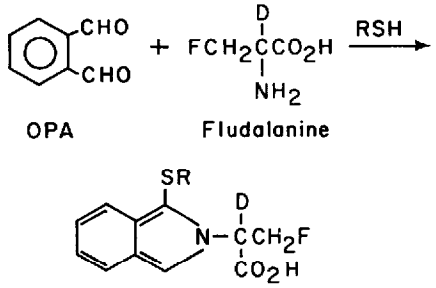

Fig. 2. *o*-Phthalaldehyde reaction with fludalanine and a mercaptan.

column derivatization [5, 6]. An HPLC assay is described for fludalanine that involves post-column derivatization* with *o*-phthalaldehyde (OPA) and 2-mercaptoethanol (Fig. 2). Optimal conditions for this post-column, packed-bed chemical reactor are described.

EXPERIMENTAL

Reagents

Sodium dodecyl sulfonate and OPA were obtained from Regis. Fludalanine, 3,3-difluoro-D-alanine (internal standard), cycloserine and sodium pantizidone hemihydrate were synthesized at Merck Sharp & Dohme Research Labs. [1, 7].

Chromatography

The liquid chromatographic system consists of one M6000A solvent delivery

*Attempts at pre-column derivatization of fludalanine in deproteinated plasma with fluorescamine, ninhydrin or *o*-phthalaldehyde (OPA) were not successful. It appeared that the derivatives were not resolved from biological background on reversed phase. Of the derivatizing reagents tested, OPA appeared to be the most reactive, yielding the greatest fluorescent response. Attempts were then made with different mercaptans and OPA (pre-column) to vary the S-alkyl group of the isoindole adduct of fludalanine in order to resolve it from background (Fig. 2). Selectivity, however, was not achieved and many of the mercaptans commercially available were not suitable (e.g. insolubility). Therefore, a method was developed involving the initial chromatography of fludalanine followed by post-column derivatization.

system for the mobile phase, a Model 710B autosampler (WISP), a Model 720 system controller and an M730 Data Module, all from Waters Assoc. The OPA reagent is delivered by a Perkin-Elmer Series 10 liquid chromatograph. The effluents from the analytical column and from the reagent pump mix in a low internal volume tee (1/16 in.) from Scientific Science and exit into a packed-bed reactor. The reactor consists of a stainless-steel column (25 cm × 4.6 mm I.D.) packed with 40-μm glass beads from Whatman. It is heated at 40°C (Temperature Control Unit, Model III, Elilex Labs.). The effluent from the reactor is monitored by a Perkin-Elmer 650-10S fluorescent detector (excitation wavelength 340 nm, emission 455 nm).

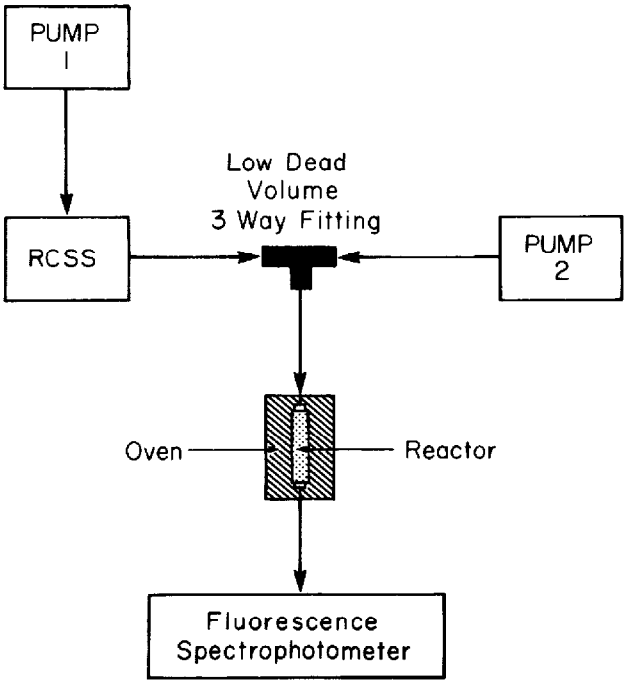

Fig. 3. HPLC set-up with a post-column reactor.

A diagram of the post-column system is shown in Fig. 3. The reagent is pumped through the high-sensitivity damper at a flow-rate of 1 ml/min to the tee. The mobile phase flow-rate is 2 ml/min through a Radial Compression Separation System (RCSS): an RCM-100 containing a Guard-Pak C_{18} and a Radial-Pak C_{18} cartridge (10 cm × 8 mm I.D., 5 μm particle size) from Waters. Radial-Pak C_{18} is LiChrosorb C_{18} (10 μm particle size), irregular packing.

The mobile phase contains 50 mg of sodium dodecyl sulfonate, 100 ml of methanol and 2 ml of 85% orthophosphoric acid. The solution is diluted to 1000 ml with water and titrated to pH 2.5 with 1.0 *M* potassium hydroxide. The solution is then filtered (0.45-μm Nylon-66 membrane filters).

The derivatizing reagent is a mix containing OPA and 2-mercaptoethanol in a borate buffer: 1 g of OPA and 0.5 ml of 2-mercaptoethanol are dissolved in 10 ml of 100% ethanol. A borate buffer is prepared with 3.0 g boric acid, 160 ml of 1 *M* potassium hydroxide diluted with water to 1 l. It is filtered (0.45-μm

Nylon-66 membrane filters). The buffer and the alcoholic solution are mixed and kept under a helium atmosphere in an amber reservoir.

The analytical column is conditioned sequentially with methanol (200—300 ml), methanol—water (1:9) and the mobile phase. The column is equilibrated when the retention time of fludalanine has stabilized (12—24 h).

Sample clean-up

Plasma. A 1-ml aliquot is mixed with 50 μl of difluoroalanine solution (0.90 mg/ml). The aliquot is transferred to a Centriflo ultrafilter (CF50A from Amicon) and centrifuged for about 15 min at 657 *g* (Dynac II centrifuge). The filtrate is analyzed.

Urine. A 0.3-ml urine aliquot is mixed with 25 μl of difluoroalanine solution (10 mg/ml). The aliquot is mixed with 2 ml of acetonitrile and loaded onto a dry silica Sep-Pak (Waters Assoc.). The mini-column is washed with 3 ml of acetonitrile, and fludalanine is eluted with 1.0 ml of water. Any silica in the eluate is centrifuged to the bottom of the receiving tube (about 10 min, 657 *g*) before analysis.

Standard calibration curves

Stock solutions of fludalanine and internal standard are prepared in water (kept cold during storage). Standards are mixed by diluting aliquots from an aqueous fludalanine stock solution (2 mg/ml) to appropriate working concentrations with water. Of each working stock solution 100 μl are mixed with 1.0 ml of plasma to achieve concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 10 and 20 μg/ml; for urine, 30 μl of each working stock solution are mixed with 300 μl of urine for concentrations of 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100 and 200 μg/ml.

RESULTS AND DISCUSSION

Post-column reaction

The post-column HPLC system for fludalanine detection (Fig. 3) involves mixing the eluate from the analytical column and the OPA reagent in a tee and then passing the mixture through a packed-bed column reactor to form a fluorescent isoindole (Fig. 2). The bed reactor consists of an empty Whatman column (25 cm × 4.6 mm I.D.) packed-dry with 40-μm glass beads. The residence time for fludalanine in the reactor is 0.5 min. The post-column reaction with fludalanine at room temperature appears to be incomplete. This was observed by stopping the flow of the reagent and the mobile phase during the elution of derivatized fludalanine from the reactor into a Schoeffel fluorometer (Fig. 4) and following the detector's response with time. The fluorometric response slowly increased from the level observed when the flow was stopped (fludalanine and OPA continued to react in the cell of the fluorometer) to a new level in 20.8 min. From this level, the response dropped only 5% over 60 min, revealing a stable fluorescent derivative.

Conditions of the post-column reactor were studied in order to optimize the reaction yield and peak resolution of fludalanine: comparisons were made of the packed-bed reactor's length, inner diameter, bead characteristic and temperature (Table I). Basically, peak width at one-half peak height remained

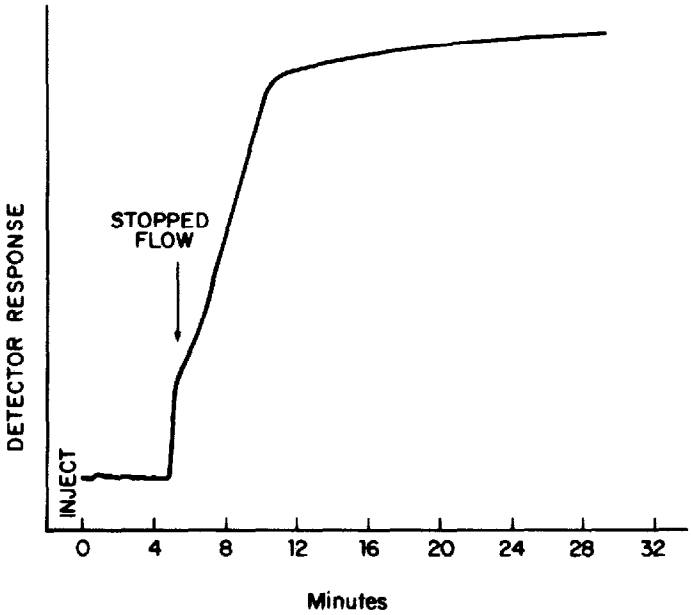

Fig. 4. A profile of the OPA-SH—fludalanine reaction with time.

TABLE I

CHARACTERISTICS OF PACKED-BED CHEMICAL REACTORS AT ROOM TEMPERATURE

Reactor	Length (cm)	I.D. (mm)	Residence time (min)	Peak height (cm)	Peak width (cm)	Peak area*
Glass beads						
1	25.0	4.6	0.50	9.2	0.6 (0.6864595)**	1.00
2	50.0	4.6	1.0	13.2	0.7 (8130531)	1.79
3	25.0	2.0	0.20	5.05	0.6 (6703337)	0.55
*Silanized glass beads****						
4	25.0	4.6	0.53	8.5	0.6 (6611166)	0.89

*The peak areas were normalized using the glass-bead reactor No. 1.
**Integrated area from a Waters data module divided by peak height in cm.
***The column was packed dry with the glass beads. Surfasil (Pierce) was forced through the column; it remained in the column at room temperature for ca. 1 h and was subsequently removed from the column with dichloromethane and ethanol. The above was repeated. Finally, the column was cleaned with water.

constant for all variations of the column make-up, except when the column was doubled in length. Also the detector response due to the post-column fludalanine reaction seems to be proportional to the residence time of the reaction in the column. A plot of the response against residence time for each of the different glass-bead columns is linear, $y = 0.243 + 1.55x$ ($r^2 = 0.9998$).

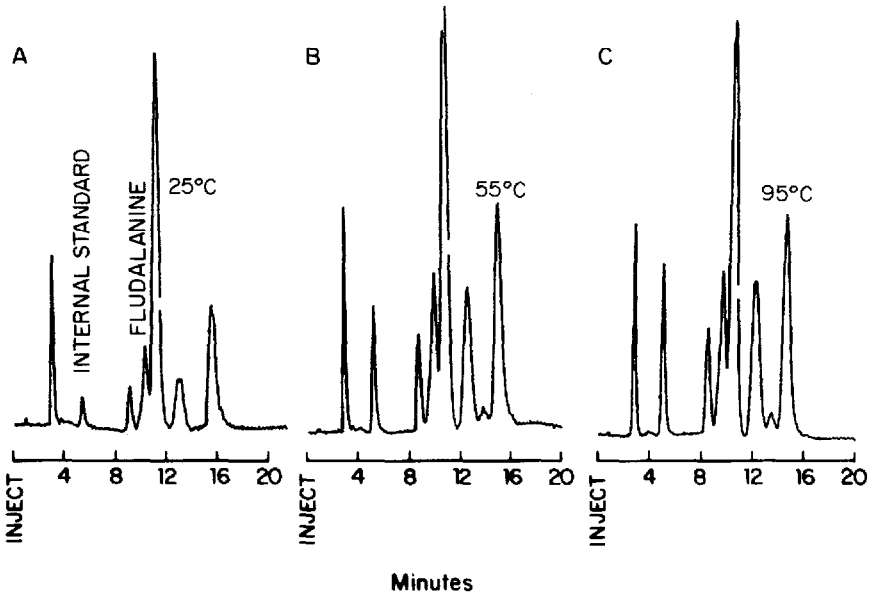

Fig. 5. Chromatograms of a plasma sample containing fludalanine (25 μg/ml) and internal standard. (A) The sample was passed through the packed-bed reactor at 25°C; (B) the same sample passed through the reactor at 55°C; (C) the same sample as the above at 95°C.

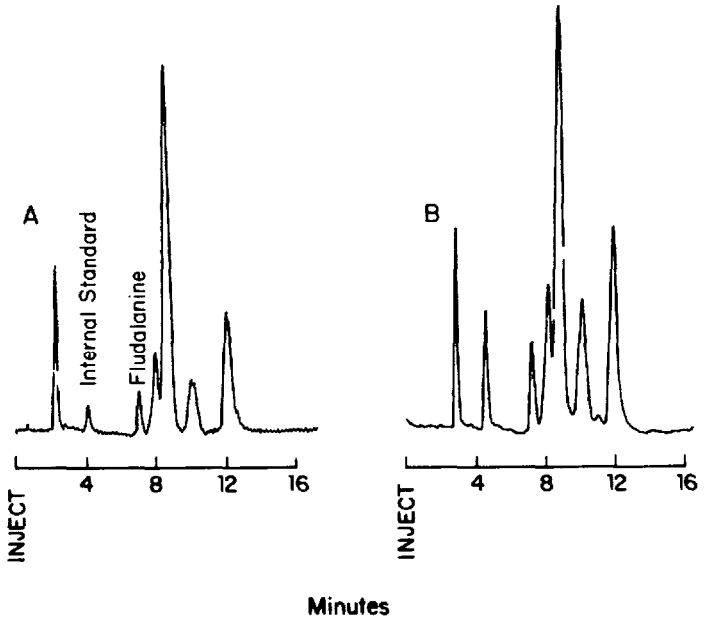

Fig. 6. Chromatograms of a plasma sample containing fludalanine (25 μg/ml and internal standard. (A) The sample was passed through a packed-bed reactor of 25 cm × 4.6 mm I.D.; (B) the same sample passed through a reactor of 50 cm × 4.6 mm I.D.

However, the response from the silanized glass-bead column compared to the regular glass-bead column was lower and gave an unexpectedly longer residence time. Raising the temperature of the reactor from 25°C to 95°C gave a

logarithmic plot of response versus temperature. At 95°C, the peak height was increased by 2.3 times. Peak width remained constant. Chromatograms of plasma standards in Figs. 5 and 6 support the above observations. Raising the temperature of the reactor from ambient improved the peak response of fludalanine but not its resolution from plasma background. The peak band width at one-half height remained the same. Doubling the length of the bed reactor improves the detector's response for fludalanine and internal standard but not resolution.

TABLE II

CHARACTERISTICS OF A PACKED-BED REACTOR VERSUS A TUBULAR COIL

Mobile phase and reagent flow-rates are 2 ml/min and 1 ml/min, respectively. Chart-speed 2 cm/min.

Reactor	Residence time (min)	Peak height (cm)	Peak width at ½ peak height (cm)	Peak area*
PTFE coil**	0.53	7.8	0.8 (8846373)***	1.09
Bed reactor§	0.50	9.2	0.6 (6864959)	1.00

*Peak areas are normalized. The areas are integrated by a Waters data module.
**Tubular column, 510 mm × 0.5 mm I.D., 62 turns with a diameter of 2.54 cm.
***Integrated area from a Waters data module divided by peak height in cm.
§Packed column, 250 mm × 4.6 mm I.D.

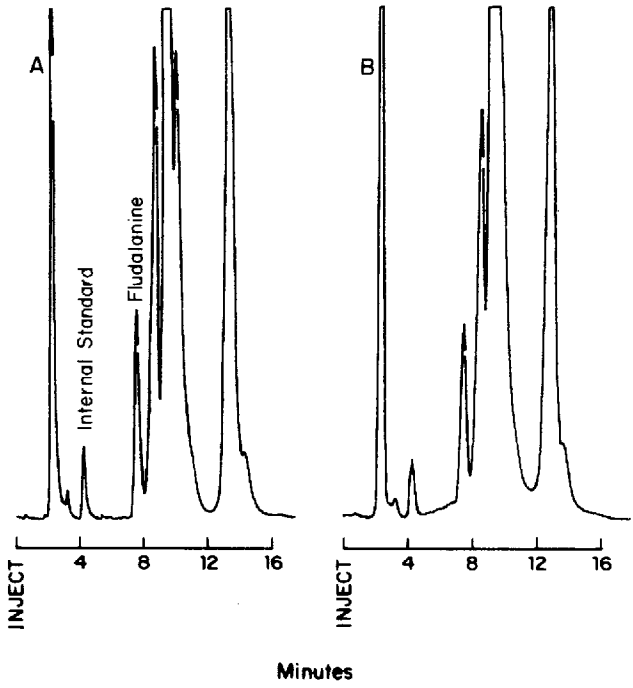

Fig. 7. Chromatograms of a plasma sample containing fludalanine (5.5 μg/ml) and internal standard. (A) The sample was passed through a packed-bed reactor (25 cm × 4.6 mm I.D.); (B) the same sample passed through a PTFE coil (510 cm × 0.5 mm I.D.).

A comparison was made of the packed-bed reactor with a PTFE, tubular coil (510 mm × 0.5 mm I.D., residence time of 0.5 min) with respect to reaction yield and peak broadening of fludalanine. Peak widths measured at one-half peak height (in cm) revealed broader peaks with the coil reactor (Table II and Fig. 7). The detector response of the coil was comparable to the packed-bed reactor. Precision of fludalanine at 2 μg/ml using the bead reactor was 6.3% (n = 5); using the coil, 6.9% (n = 5). Recently, Kratos introduced a reactor unit consisting of epoxy-supported PTFE tubing with a unique configuration (encased in a metal cylinder). The Kratos' unit (1.5 ml) produced sharper peaks but lower reaction yield when compared to the packed-bed reactor (Table III).

TABLE III

KRATOS' TUBULAR REACTOR VERSUS A PACKED-BED

Reactor	Peak height (cm)	Band width (cm)	Area*
Tubular**	11.7	0.30	$3.05 \cdot 10^8$
Packed-bed column	11.2	0.35	$4.21 \cdot 10^8$

*Generated from a Waters Data Module.
**Dead volume of 1.5 ml.

Chromatography

An ion-pairing agent in the mobile phase is needed to increase the retention time of fludalanine significantly beyond the void volume using a radially compressed, reversed-phase column*. The ion-pairing agent dodecyl sulfonate in methanol and water provides a chromatographic window for fludalanine and its internal standard difluoroalanine, clearly separated from the plasma or urine background (Fig. 8 and 9). Shorter-chain-length alkylsulfonates do not give the requisite separation.

The assay does not give a detector response for the co-administered prodrug of cycloserine, pentizidone. Cycloserine and the degradation products of cycloserine and fludalanine do not interfere. Plausable by-products of fludalanine via loss of deuterium fluoride are D-serine and pyruvic acid [8]. Fludalanine elutes before serine and most other amino acids (e.g. alanine, glycine, aspartic acid, etc.) Cycloserine and the dimer of cycloserine, which is a by-product [9], elute much later.

Assay parameters

The plasma assay for fludalanine is linear from 0.25 to 20 μg/ml with a least-squares analysis of $r^2 = 0.9998$, $y = 0.0213 + 0.0213x$. The urine assay is linear from 0.5 to 200 μg/ml with $r^2 = 0.9997$, $y = 0.0099 + 0.0138x$.

Intra-day reproducibility over the above plasma range has a mean value of 6.14 ± 2.0%; for urine standards, the mean is 5.18 ± 2.25% (Table IV). Inter-

*Stainless-steel columns of comparable or of better end-capped packing (e.g. Altex Ultrasphere-IP ODS) did not provide sufficient resolution for fludalanine from biological background.

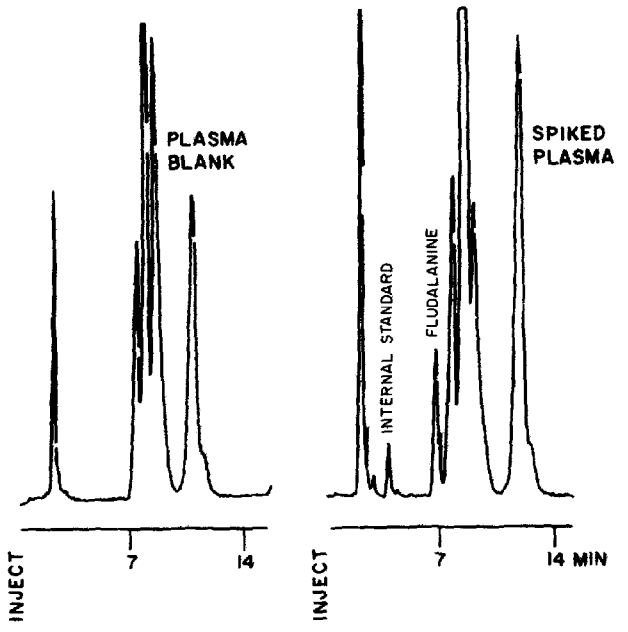

Fig. 8. Chromatograms of a plasma blank sample and of a plasma sample containing fludalanine (5.5 μg/ml) and internal standard.

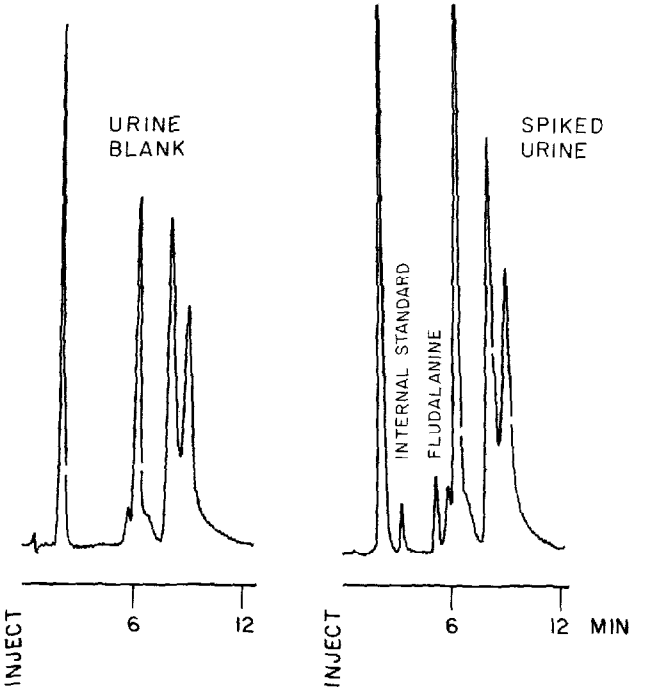

Fig. 9. Chromatograms of a urine blank and of a urine sample containing fludalanine (10.0 μg/ml) and internal standard.

TABLE IV

COEFFICIENTS OF VARIATION OF REPLICATE ANALYSES (n = 6) OF FLUDALANINE

Standard (μg/ml)	C.V. (%)
Plasma	
0.25	6.8
0.50	3.3
1.0	9.3
2.5	4.5
5.0	5.7
10	7.7
20	5.7
Urine	
0.50	7.4
1.0	7.1
2.5	5.6
5.0	5.3
10	4.4
25	1.9
50	2.0
100	4.6
200	8.3

day reproducibility for the plasma assay is 8.7% at 12.5 μg/ml; for the urine it is 8.3% at 100 μg/ml. Recovery of fludalanine from plasma by ultrafiltration prior to HPLC is 48.9 ± 3.0%; recovery from urine via adsorption chromatography prior to HPLC is 66.5 ± 4.3%. The values are based on peak heights of treated biological standards compared to aqueous standards.

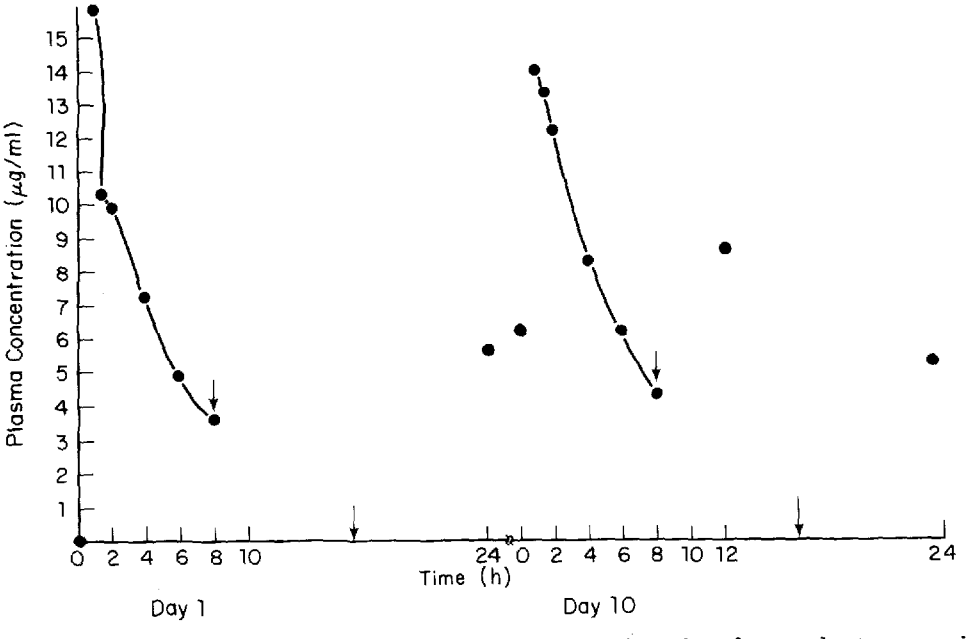

Fig. 10. Human plasma levels of fludalanine plotted against time from volunteers receiving 500 mg fludalanine orally every 8 h for ten days.

Application

The assay methodology for fludalanine has been applied to samples from a multiple-dose study. Male volunteers were orally administered 500 mg of fludalanine three times a day for ten days. Blood samples were collected on day 1 and on day 10 up to 24 h. Plasma concentrations for one volunteer ranged from just under 16.0 μg/ml at 1 h post dose to 3.6 μg/ml at 8 h on day 1 (Fig. 10). The apparent half-life was about 3.4 h.

The urine concentrations from the same volunteer during the first 8 h of collection (time intervals: −1—0, 0—1, 1—2, 2—4, 4—6, 6—8) ranged from 34.1 to 173.0 μg/ml. Dose recovery was 34.1%. During the remaining treatment period (time intervals: days 2—9 with 0—24 h collections; day 10 with −1—0, 0—1, 1—2, 2—4,, etc. collections) concentrations ranged from 13.2 to 375 μg/ml. Dose recovery at steady state was 53.1%.

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