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Determination of fluspirilene in human plasma by liquid chromatography–tandem mass spectrometry with electrospray ionisation

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Abstract

An ultra-sensitive method for the determination of fluspirilene in plasma was established, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The samples were extracted with hexane/isoamyl alcohol, separated on a Phenomenex Luna C_{18} 5 μ 150×2.1 mm column with a mobile phase consisting of methanol–water–acetic acid (600:400:1) at a flow-rate of 0.3 ml/min. Detection was achieved by a Finnigan Matt mass spectrometer (LCQ) at unit resolution in full scan mode scanning the product ion spectrum from *m/z* 130–500 and monitoring the transition of the protonated molecular ion at *m/z* 476.2, to the sum of the largest product ions *m/z* 371, 342 and 274 (MS–MS). Electrospray ionisation was used for ion production. The mean recovery for fluspirilene was 90% with a lower limit of quantification of 21.50 pg/ml using 1 ml plasma for extraction. This is the first chromatographic method described for the determination of fluspirilene in plasma that is accurate and sensitive enough to be used in pharmacokinetic studies.

Keywords: Mass spectrometry; Fluspirilene

1. Introduction

Fluspirilene, 8-[4,4-bis(4-fluorophenyl)butyl]-1-phenyl-1,3,8-triazaspirol [4.5] decan-4-one is a diphenylbutylpiperidine neuroleptic used in the treatment of schizophrenia. It has similar pharmacological properties to those of the phenothiazine compounds, e.g., chlorpromazine. The usual dose is 2 mg intramuscular (i.m.) weekly with resultant maximum plasma concentrations of about 200 pg/ml.

Fluspirilene is metabolised on release from the injection site and the main metabolite, which is 4,4-bis(4-fluorophenyl)-butyric acid obtained by *n*-dealkylation, is excreted in the urine. The elimination half-life of fluspirilene after i.m. injection is reported to be approximately three weeks [1].

No reference to any chromatographic method using conventional detectors for the determination of fluspirilene in plasma could be found in the literature. Aderjan et al. [2] described a chromatographic method for the separation of some diphenylbutylpiperidines and the subsequent determination of fluspirilene by radio-immunoassay. To construct a pharmacokinetic profile after a single dose of fluspirilene thus presents a considerable analytical challenge. Ultra-sensitive analysis of drugs

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in the lower pg/ml levels in biological fluids however, can be performed on a mass-selective detector with tandem mass spectrometry (MS–MS) capabilities in tandem with liquid chromatography (LC) such as the Finnigan Mat LCQ MS^n.

2. Experimental

2.1. Materials and chemicals

A Phenomenex Luna C_{18} 5 μ, 150×2.1 mm column (Phenomenex, Torrance, CA, USA) was used for separation at a flow-rate of 0.3 ml/min and injecting 20 μl onto the column. The mobile phase was delivered by a Hewlett-Packard Series 1050 pump and the samples injected by a Hewlett-Packard Series 1050 auto-sampler (Hewlett-Packard, Palo Alto, CA, USA). Detection was performed by a Finnigan Mat LCQ MS^n detector (Finnigan Mat, San Jose, CA, USA) using electrospray ionisation (ESI) for ion production.

Acetic acid and isoamyl alcohol (Pro-Analysi), were obtained from Merck (Darmstadt, Germany); hexane and methanol (Burdick & Jackson, High Purity) were obtained from Baxter, USA, and sodium hydroxide (analytical-reagent grade) from Fluka (Buchs, Switzerland). All chemicals were used as received. Water was purified by RO 20SA reverse osmosis and Milli-Q polishing system (Millipore, Bedford, MA, USA).

Fluspirilene, $C_{29}H_{31}F_2N_3O$, (Fig. 1) was supplied by Schweizerhall.

2.2. Extraction procedure

Fluspirilene standard solutions were made up in methanol and used immediately to spike plasma and discarded thereafter. Calibration standards and quality control standards were prepared in normal human plasma and by spiking a pool of normal plasma which was then serially diluted with normal blank plasma to attain the desired concentrations (12–833 pg/ml). The calibration standards and quality control standards were aliquoted into tubes and stored under the same conditions as the trial samples; approximately −20°C in the same freezer as the subjects' samples.

To 1 ml plasma in a 5-ml amber ampoule was added 250 μl of 1 *M* sodium hydroxide and 3 ml of a 4% solution of isoamyl alcohol in hexane. The sample was vortex mixed for 2 min and centrifuged at 3300 *g* for 5 min at 10°C.

The aqueous phase was frozen at −30°C on a Fryka Polar cooling plate (Kältetechnik, Esslingen), the organic phase decanted into a clean amber glass ampoule and evaporated under vacuum on a Savant SpeedVac (Savant Instruments, NY, USA) rotary evaporator at ambient temperatures.

The residue was reconstituted in mobile phase (200 μl), the solution transferred to an autosampler vial containing a 200 μl glass insert for injection onto the analytical column. To prevent any possible degradation of the analyte, samples were kept cold at approximately 2°C on the autosampler by circulating refrigerated water with a Lauda RM-6 cooling device (Lauda Dr. R. Wobser, Lauda-Königshofen).

2.3. Liquid chromatography

All chromatographic solvents were sparged with helium before use. Chromatography was carried out at ambient temperature at a flow-rate of 0.34 ml/min with methanol–water–acetic acid (600:400:1, v/v) as mobile phase. The column outlet was connected to a 100-μm I.D. fused-silica capillary which transferred the whole eluent into the ion source.

2.4. Mass spectrometry

Electrospray ionisation was performed in the positive mode with nitrogen as the nebulizing (78 units) and auxiliary gas (19 units). For tuning, a T-piece was installed in the flow-line from the high-performance liquid chromatography (HPLC) system before the connection to the silica capillary, and connected to the syringe pump of the instrument via a piece of polyether ether ketone (PEEK) tubing. The response of the instrument for fluspirilene was optimised by injecting a constant flow of a solution of the drug in mobile phase into the stream of mobile phase eluting from the column. The response was optimal with a spray voltage setting of 3.49 kV and a spray current of 0.83 μA. The heated capillary voltage was set at 3.07 V and the temperature to 196°C.

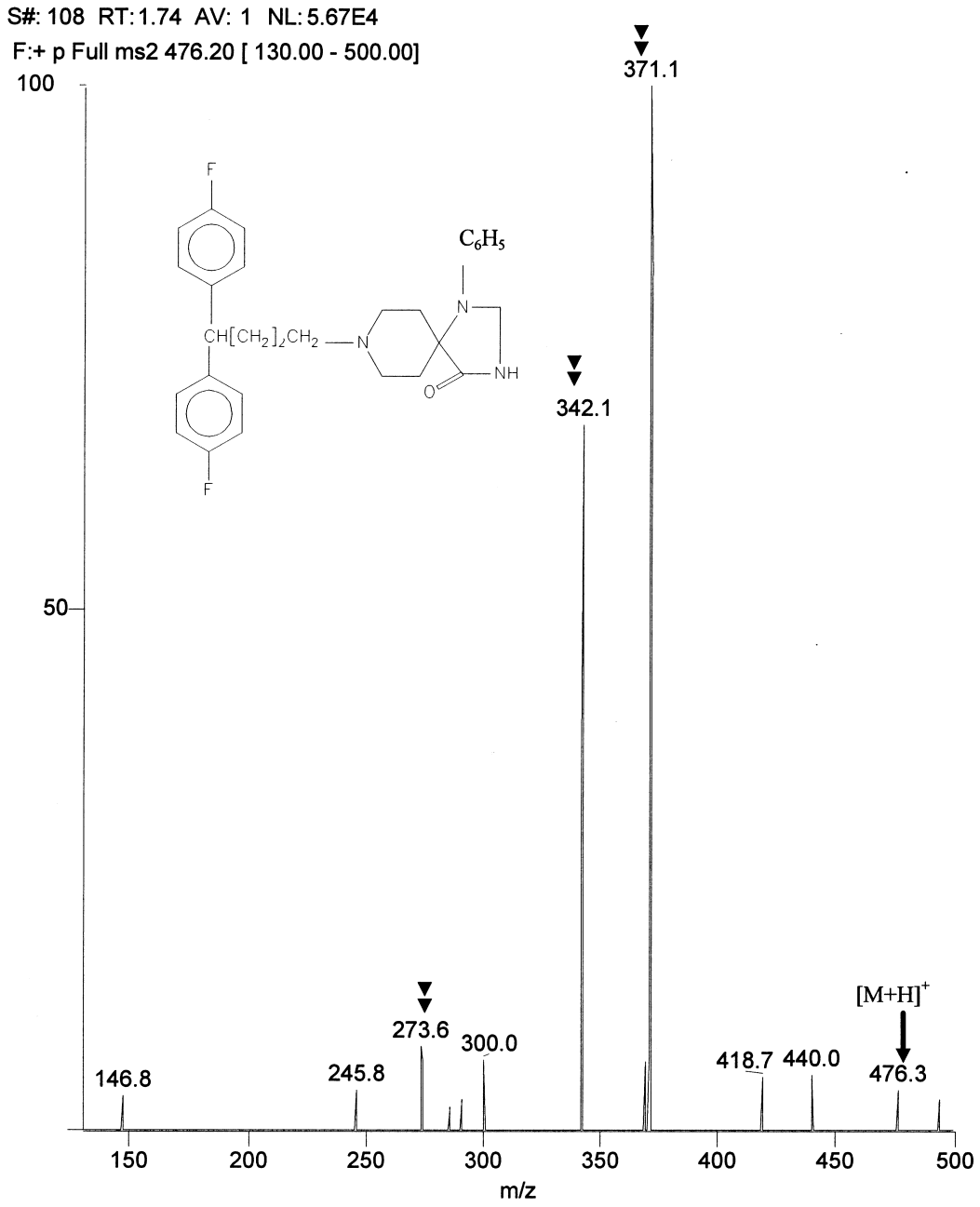

Fig. 1. Mass spectrum showing the structure of fluspirilene and the product ions formed from m/z 476 after collision (MS–MS).

The instrument was operated at unit resolution in full scan MS–MS mode, scanning the product ion spectrum from m/z 130–500. The transition of the protonated molecular ion at m/z 475.6 for fluspirilene to the largest product ions were m/z 371, 342 and 274. The maximum inject time was set at 600 ms and the total microscans at 1. The isolation width was 2 a.m.u. and the relative collision energy

set at 15%. The LCQ was interfaced to a computer workstation running Finnigan Mat LCQ Navigator software.

2.5. Validation

The method was validated by analysing plasma quality control samples five times at six different concentrations i.e., 754, 417, 208, 62.4, 46.5, 31.2 and 15.6 pg/ml to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve containing eight different concentrations spanning the concentration range (10.7–829 pg/ml). Calibration graphs were constructed using a weighted linear regression of the sum of the drug peak-areas of the daughter ions (m/z 371, 342 and 274) for fluspirilene versus nominal drug concentrations.

Specificity was determined by analysing ''blank'' biological fluids from six different sources without the addition of the internal standard to determine possible interference with the analyte and the internal standard.

Absolute recoveries of analyte and internal standard were determined in triplicate in normal plasma by extracting drug-free plasma samples spiked with fluspirilene. Recoveries were calculated by comparison of the analyte peak areas of the extracted samples with those of the unextracted response standard mixtures representing 100% recovery. The recovery was calculated using the response standard since no difference in ionisation between extracted samples and pure solutions was observed.

3. Results and discussion

The mean absolute recoveries of analyte determined in triplicate at 30.8, 208 and 417 pg/ml were 90.3%, 88.2% and 88.8%, respectively.

The lower limit of quantification (LLOQ) was initially determined from the data obtained for the assayed quality controls during pre-study validation, since these data included determinations of the analyte at concentrations close to the limit of detection. The LLOQ is defined as that concentration of the fluspirilene which can still be determined with acceptable precision (C.V.<20%) and accuracy (bias<20%). This limit was reappraised during the performance of the assay with actual clinical study samples. After all the clinical study samples had been analysed the limit of quantification was finally set at a value which was determined by the performance of the procedure with the quality controls which were processed with each batch of samples run. This is considered to be a more objective reflection of the assay performance under clinical study conditions than the validation data alone. The LLOQ was finally set at 21.5 pg/ml, i.e., at the value determined during the pre-study validation. Results from the intra-day validation assays indicate a valid calibration range of 21.5–829 pg/ml. The LLOQ was set at 21.5 pg/ml, i.e., at the lowest concentration of the calibration standard. Table 1 shows the quality control data obtained during the validation of the method, while Table 2 depicts the intra-day back calculated calibration standards, showing a valid calibration range from 21.5 to 829 pg/ml.

On-instrument stability was inferred from intra-day quality control data obtained during the pre-study validation. No significant degradation could be detected in the cooled samples left on the auto-sampler for at least 12 h (Fig. 2).

Plasma samples containing fluspirilene at concentrations of 749 pg/ml and 46.10 pg/ml were stored at −20°C for eight months and compared to freshly prepared standards and quality controls. No evidence of fluspirilene degradation was found in these samples (Table 3).

Due to the high specificity of MS–MS detection, no interfering or late eluting peaks were found when

Table 1
Summary of quality control results of fluspirilene in human plasma as obtained during the validation (inter-day variation)

Added concentration (pg/ml)	Fluspirilene (n=5) mean concentration found (pg/ml)	R.S.D. (%)	Accuracy (bias, %)
15.6	14.9	1.8	−4.2
31.2	29.1	5.3	−6.8
46.5	50.7	7.9	9.1
62.4	66.2	6.8	6.1
208	216	10.1	3.8
417	417	2.8	0.0
754	742	11.5	−1.6

Table 2
Summary of back-calculated calibration standards concentrations of fluspirilene (intra-day variation) showing the repeatability of the method (weighted linear regression)

Nominal (pg/ml)	21.5	30.2	42.6	105	231	342	465	Regression	Slope	Intercept
	22.3									
	R	30.5	40.8	102	227	372	443	0.99532	634	851
	24.8									
	22.1	27.2	42.9	87	255	338	462	0.99240	222	−611
	19.5									
	16.8	37.3	38.7	117	271	321	438	0.98379	188	926
	23.1									
	22.8	31.2	37.4	85	267	367	425	0.98306	132	−606
	25.2									
	R	29.4	42.8	91	231	294	525	0.97998	119	−514
	23.1									
	R	32.8	36.0	101	244	330	470	0.99670	91	523
	26.3									
	20.2	29.0	42.2	89	238	333	481	0.99489	98	−331
Mean	22.4	31.1	40.1	96	248	336	463	0.98945	212	34
% Bias	4.1	2.8	−5.8	−8.6	7.2	−1.7	−0.4			
R.S.D. (%)	11.6	9.7	6.4	10.9	6.4	7.4	6.6			

R=Rejected (outliers).

chromatographing the blank plasma extracts from six different sources.

Different concentrations of acetic acid, formic acid and volatile buffers were tested for optimum ionisation of the analytes. It was found that 0.1% acetic acid gave the best results.

Fig. 1 shows the single parent to product ion mass spectrum (MS–MS) of fluspirilene acquired with the

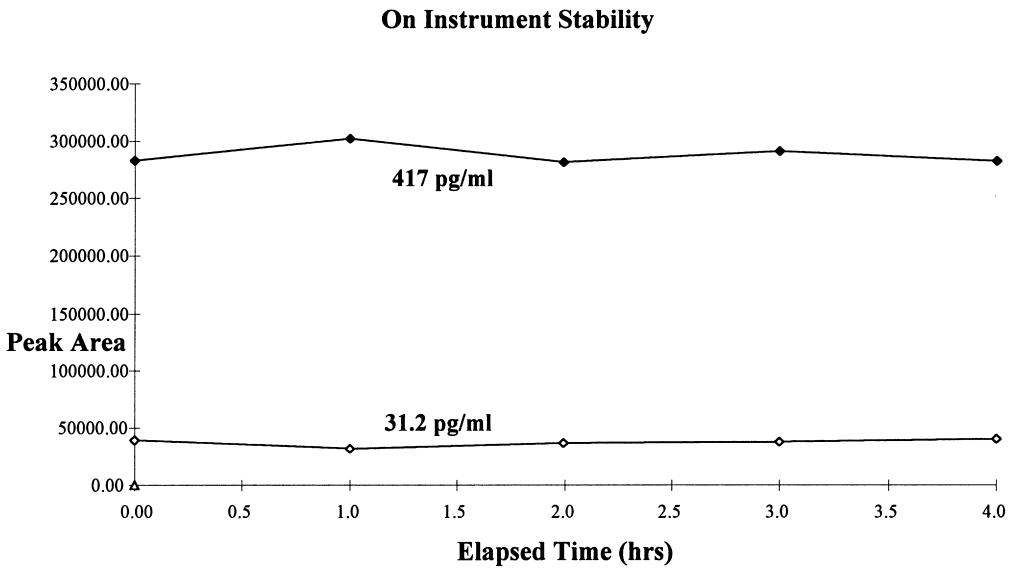

Fig. 2. On-instrument stability as depicted when samples containing 417 pg/ml and 31.2 pg/ml were injected approximately every hour.

Table 3
Stability samples analysed with fresh standards after stored at −20°C for eight months

Concentration 1 (Nominal conc.)	Measured concentration [Conc. 1 (−20°C)]	Concentration 2 (Nominal conc.)	Measured concentration [Conc. 2 (−20°C)]
749.00	609.60	46.10	43.46
749.00	771.88	46.10	31.25
749.00	794.92	46.10	36.64
749.00	801.26	46.10	34.97
749.00	755.84	46.10	46.36
Mean	746.70		38.54
S.D.	78.77		6.22
R.S.D. (%)	10.55		16.15
% Nominal	99.69		83.59

abundant product ion at m/z of 371 and ions of lower abundance at m/z of 342 and 273.8.

Quantitation was achieved at unit resolution in full scan MS–MS mode scanning the product ion spectrum from m/z 130–500 and monitoring the transition of the protonated molecular ion at m/z 476.2 for fluspirilene, to the sum of the largest product ions m/z 371, 342 and 273.8.

Typical retention times were 1.40 min for fluspirilene. A high concentration of methanol had to be added to shorten the retention time of fluspirilene and to prevent peak tailing. The total chromatography time of 3 min, made it possible to analyse a large number of samples in a relative short period of time. The retention time for fluspirilene had to be kept as short as possible to obtain peaks that was still above a signal-to-noise level of 5. Longer retention times resulted in peaks that were tailing which resulted in higher limits of quantification, which were unacceptable for pharmacokinetic studies. Although the k' value is less than 2, a compromise had to be made between possible matrix interference and the limit of quantification. To obtain this care had to be taken to introduce ''clean'' components into the ionisation source. This was achieved by performing liquid–liquid extractions with hexane followed by chromatographic separation of the extract in order to minimise possible matrix effects on ionisation which

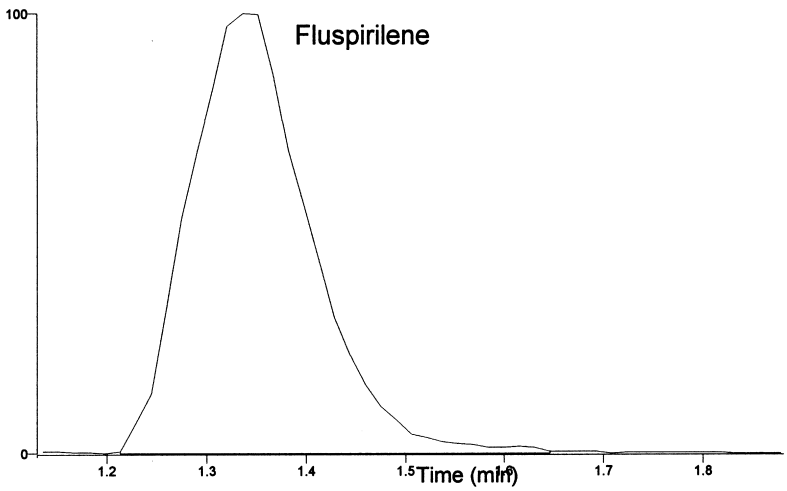

Fig. 3. High-performance liquid chromatogram of a calibration standard containing 231 pg/ml of fluspirilene in plasma.

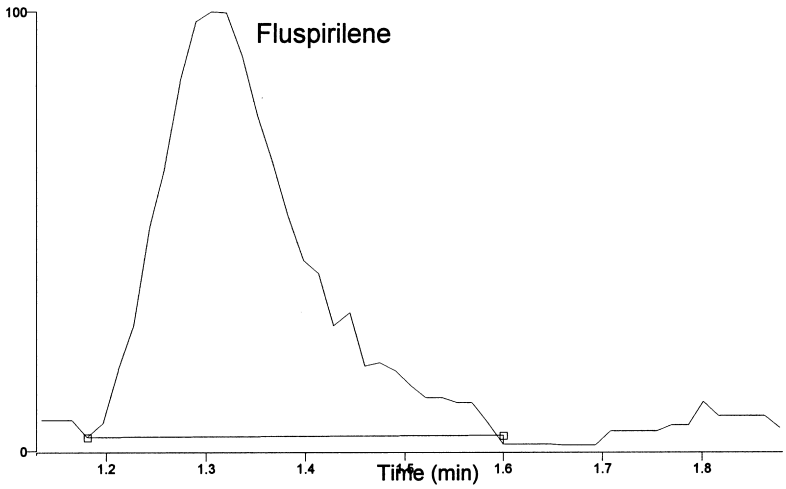

Fig. 4. High-performance liquid chromatogram of a calibration standard containing 21.51 pg/ml of fluspirilene in plasma.

could occur with direct flow-injection of the supernatant obtained from protein-precipitated samples. Since the calibration curves obtained were fairly linear it is doubtful that any matrix effects occurred under the conditions described.

Figs. 3 and 4 show representative chromatograms obtained at 231 pg/ml and 21.5 pg/ml (LLOQ), while Figs. 5 and 6 depict chromatograms from a subject sample and a blank plasma extract, zoomed in to show the absence of any interference.

To increase the extraction yield, 4% isoamyl alcohol had to be added to the hexane. Back-extractions of the analytes into HCl or acetic acid to save on evaporation time and to obtain possible cleaner extracts, gave very low recoveries and could therefor not be used. Sample cleanup on different solid-phase extraction cartridges also gave very low recoveries. Under the conditions described a mean recovery of nearly 90% was obtained for fluspirilene.

Dimethothiazine ($m/z=391.5$) was originally used

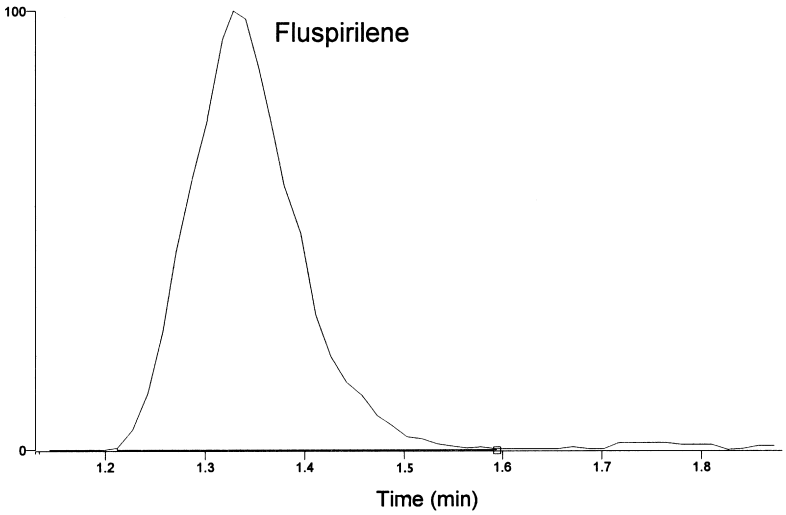

Fig. 5. High-performance liquid chromatogram of a subject sample containing 168.5 pg/ml of fluspirilene in plasma.

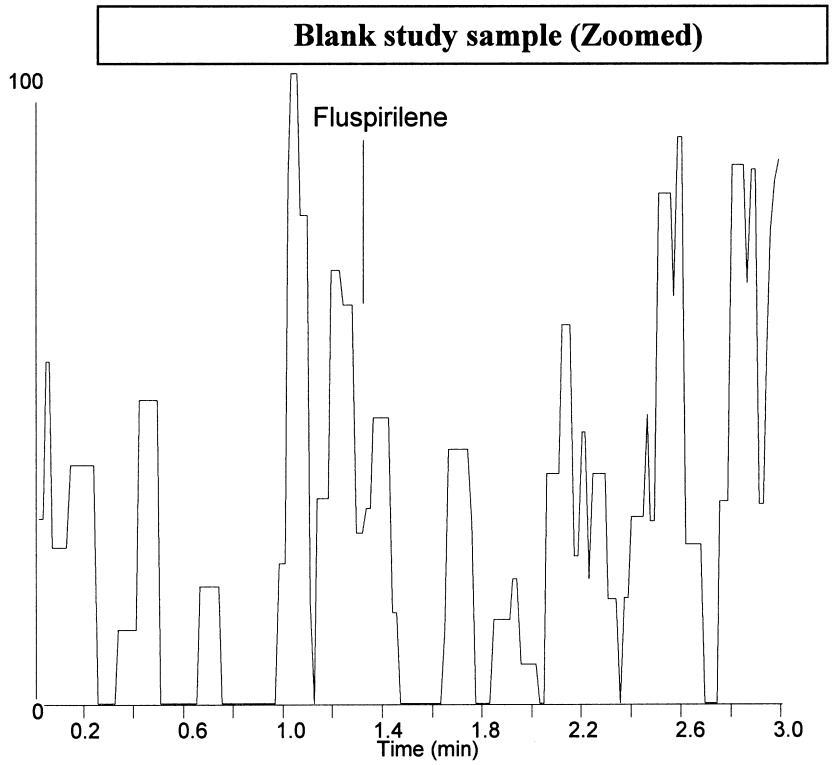

Fig. 6. High-performance liquid chromatogram of a blank plasma extract zoomed in to show the absence of any interference.

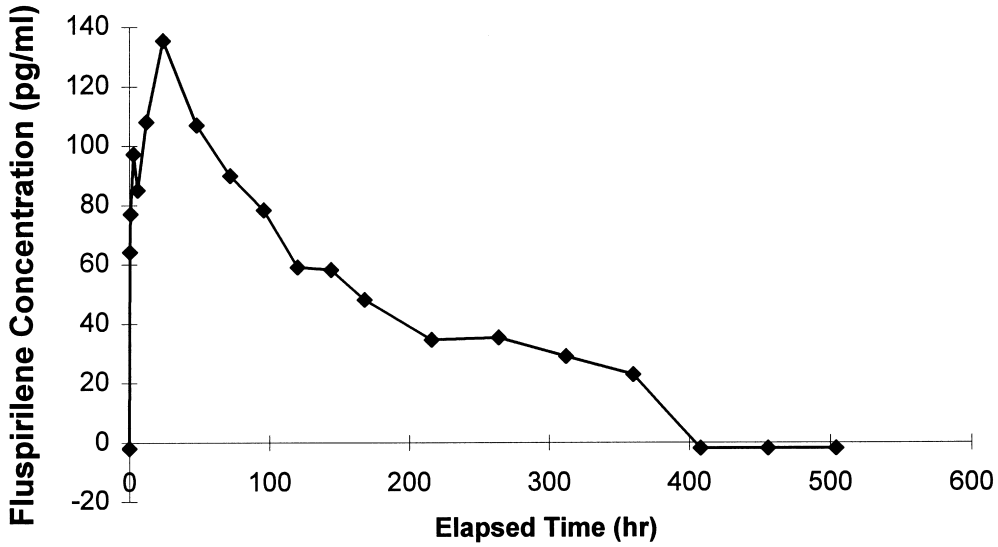

Fig. 7. Representative fluspirilene plasma time profile as obtained after a single 2-mg intramuscular dose.

as internal standard but better results were obtained without the addition of the internal standard. The extraction, chromatography and ionisation of the analytes were constant enough so that the method could be used without an internal standard.

The method was employed to analyse plasma samples containing fluspirilene obtained after a single i.m. dose of 2 mg in 26 healthy volunteers. Concentration vs. time profiles could be constructed for up to 15 days. The maximum plasma concentrations obtained varied between 83 and 280 pg/ml. Fig. 7 shows a typical pharmacokinetic profile of a subject after receiving a 2 mg i.m. dose of fluspirilene.

4. Conclusions

A highly sensitive and selective method for the quantification of fluspirilene in human plasma has been developed and validated. Plasma concentrations of fluspirilene could be quantified from 21.5 pg/ml to 829 pg/ml making it applicable to apply to pharmacokinetic studies where 15-day concentration vs. time profiles after a single 2 mg i.m. dose of the drug are required. This is the first chromatographic method for fluspirilene in plasma described using LC in tandem with mass spectrometry.

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