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Rapid and simple micromethod for the quantification of fluindione in human plasma using high-performance liquid chromatography

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Abstract

A new high-performance liquid chromatographic (HPLC) assay without any extraction procedure was developed for the quantification of fluindione in plasma using a 100-μl sample volume and coumarin as the internal standard. A deproteinization procedure was coupled with a reversed-phase HPLC separation using a 250×4.6 mm I.D. C_{18} column and a UV detector set at 280 nm. Peak height ratios were linear over the range 0.05 to 10 μg/ml (correlation coefficient >0.998). The method was found to be highly reproducible, as indicated by the low value obtained for the coefficient of variation: C.V.≤6.1% (n=10). The limit of quantification, estimated under the described conditions at a signal-to-noise ratio of three and with a C.V. lower than 20% for precision and accuracy, was 0.025 μg/ml. The total turnaround time was 25 min. After storage of blood samples at concentrations of 0.1, 0.5 and 2.5 μg/ml at room temperature and exposition to light for 120 h, no degradation of fluindione occurred. This micromethod is simple (no extraction step), fast and currently is being used for drug monitoring. © 1998 Elsevier Science B.V.

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1. Introduction

Fluindione (2-(*P*-fluorophenyl)-1,3-indandione) is an oral indirect-acting indanedione anticoagulant with a long half-life and inhibits the synthesis of vitamin K-dependent clotting factors. It is used in various cardiologic diseases for the prevention of thromboembolism. Recently, the results of one clinical trial on heparin–fluindione relay were published [1]. In another trial, Cazaux et al. [2] used the international normalised ratio (INR) from 46 patients to predict the daily maintenance dose of fluindione; no haemorrhagic complication was reported. A few case reports by various authors mentioned the use of fluindione in different situations: one case of neutropenia induced by acenocoumarol disappeared quickly after substitution of acenocoumarol by fluindione [3]. Adverse effects have been reported, including prolonged fever induced by fluindione in one patient [4], one case of immuno-allergic hepatitis that was probably related to fluindione [5] and a pseudomelanoma that appeared in one patient receiving fluindione and other drugs [6].

No method has been published for the direct assay of fluindione without an extraction step. Two methods have been published for assaying fluindione [7,8]: Roncato et al. [7] described a high-performance liquid chromatographic (HPLC) method for the

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determination of fluindione in human plasma using a reversed-phase column (C_{18}) with UV detection and Lofti et al. [8] described a HPLC method for the determination of thirteen anticoagulants, including fluindione, in human plasma using a reversed-phase column (C_{18}) with a diode array detector. Some other methods have been published on the determination of 4-hydroxycoumarin and other indanedione derivative anticoagulants in human plasma [9–14] using reversed-phase HPLC with UV [9–14] or fluorescence [11,12] detection. These methods can be used to assay warfarin and its four metabolites [9], acenocoumarin [10], 2-indanedione and 6-hydroxycoumarin [11], 1-indanedione and 4-hydroxycoumarin [12], coumarin and its metabolites [13], and coumarin [14].

These methods require a large volume of plasma (1–2 ml) and are time-consuming as they include an extraction step for sample clean-up (liquid–liquid [7–10,12–14] or solid-phase extraction with aluminium oxide/C_{18} column [11]).

The assay presented in this paper is a simple, rapid and sensitive HPLC micromethod for the quantification of fluindione in human plasma using a 100-μl volume of sample and without any extraction procedure.

The method has been applied to a pharmacokinetic (PK)–pharmacodynamic (PD) study in 50 patients who were administered a therapeutic dose of fluindione (usually 20 mg daily) [15]. This study was developed as a tool for the therapeutic drug monitoring of fluindione from individual PD measurements (INR, prothrombin complex activity (PCA), clotting factor VII) using the established PK–PD model and a Bayesian estimation method.

2. Experimental

2.1. Apparatus and chromatographic conditions

The fluindione assay in plasma samples was performed by HPLC using a P-1000 isocratic pump, an AS-3000 100 μl loop volume autosample injector, set to a run time of 10 min/sample, a UV-1000 variable-wavelength ultraviolet detector set at 280 nm (wavelength corresponding to the maximum absorbance of fluindione) and a PC-1000 integrator (TSP: Thermo Separation Products, Fremont, CA, USA). The separation was achieved at room temperature using a reversed-phase 250×4.6 mm I.D. Supelcosil LC-18 column (Supelco, Bellefonte, PA, USA) with 5 μm particle size packing. The mobile-phase composition was optimized to a 0.067 *M* Na_2HPO_4 buffer (adjusted to pH 7.2 with H_3PO_4) and acetonitrile (77:23, v/v) mixture. The mobile phase was filtered through a 0.22-μm filter that was purchased from Millipore (Durapore, GPWP 047, Bedford, MA, USA) and then degassed for 5 min ultrasonically. The flow-rate was set at 1.5 ml/min, with an average operating pressure of 105 bar. At the end of each chromatographic session, the HPLC system was rinsed with 200 ml of acetonitrile–deionized water (50:50, v/v).

2.2. Chemicals

Fluindione was kindly supplied by Procter and Gamble (Neuilly-sur-Seine, France). Coumarin (2*H*-1-benzopyran-2-one) (the I.S.) was kindly supplied by Boots Pharmaceuticals (Courbevoie, France).

The molecular structures of fluindione and the I.S. are shown in Fig. 1.

Acetonitrile (Lichrosolv, Merck, Darmstadt, Germany) and methanol (Uvasol, Merck) were of UV grade. Disodiumhydrogenphosphate dihydrate buffer (Na_2HPO_4; Merck) was of analytical grade. Sodium chloride (0.9% solution; Biosedra Pharma, Louvier, France) was of European Pharmacopoeia grade.

Stock solutions of fluindione and the I.S. were prepared by dissolving 10 mg equivalent free and pure base of each molecule in 10 ml of methanol; these were then stored at +4°C. The stock solutions (1 mg/ml) were found to be stable for several weeks. The working solutions (50 and 10 μg/ml) were prepared as required.

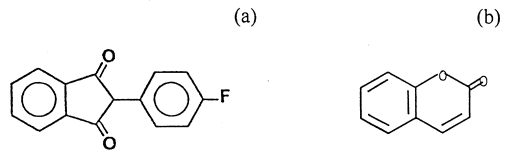

Fig. 1. Molecular structures of fluindione (a) and coumarin (internal standard) (b).

2.3. Plasma sample pretreatment

A 100-μl volume of plasma from human heparinized blood (spiked plasma used for calibration and controls, patients' plasma samples) was added to a 5-ml siliconized tube (Venoject, Terumo, Belgium) containing 50 μl of a 2 μg/ml solution of the I.S. and 100 μl of acetonitrile. The tube was vortex-mixed for 30 s and centrifuged for 10 min at 3000 *g*. A 150-μl volume of supernatant was transferred to another tube containing 150 μl of NaCl (0.9% solution). After mixing, 100 μl of the mixture were injected into the HPLC system.

2.4. Preparation of calibration curves

A calibration curve based on peak-height ratio was constructed for each assay by adding known amounts of fluindione to drug-free human plasma. Nine different spiked plasma samples, covering the concentration range 0, 0.05, 0.1, 0.25, 0.5, 1, 2, 4 and 6 μg/ml, were assayed. Each spiked plasma sample was processed as described previously. Linearity of the calibration curves was determined by linear least-squares regression analysis.

3. Results and discussion

3.1. Quantification, separation and plasma interferences

Chromatograms obtained from drug-free human plasma, spiked plasma and plasma samples from patients receiving (daily) 20 mg doses of fluindione are shown in Fig. 2. The retention times of fluindione and the I.S. were 4.2 and 8.5 min, respectively. The peaks were adequately resolved without any interference from endogenous compounds.

3.2. Linearity and limit of quantification

The calibration curve was linear over the range 0.05 to 10 μg/ml. The correlation coefficient was better than 0.998. The method was found to be highly reproducible, as indicated by the low value obtained for the C.V. (Table 1). The limit of quantification, estimated under the described conditions at a signal-to-noise ratio of three and with a C.V. of less than 20% for precision and accuracy, was 0.025 μg/ml.

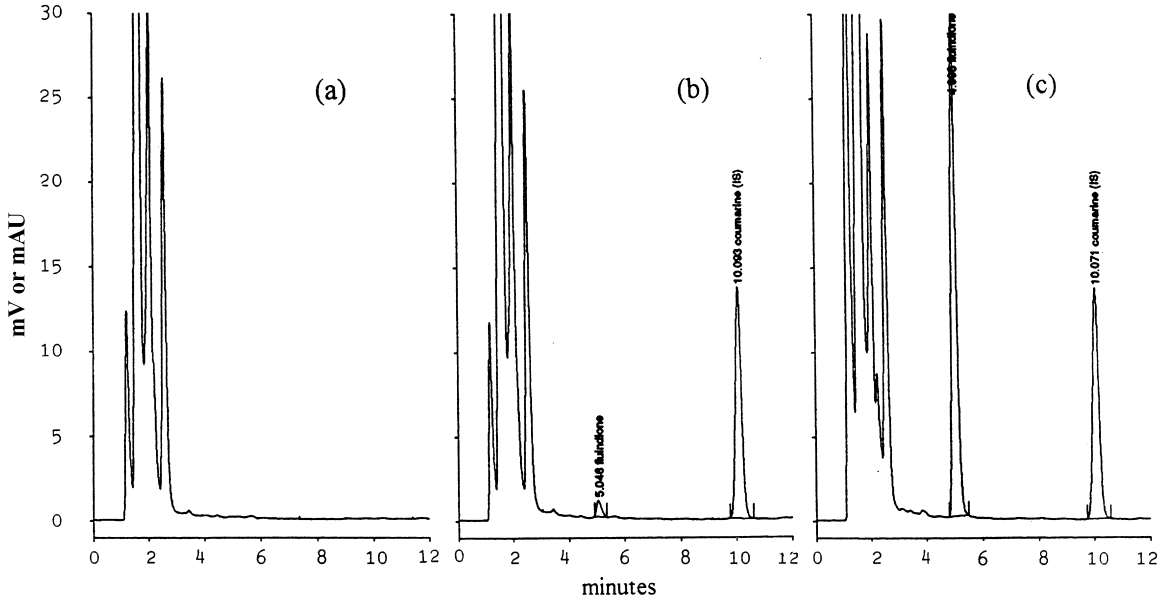

Fig. 2. Chromatograms obtained from (a) drug-free human plasma sample; (b) spiked plasma sample with 0.1 μg/ml of fluindione; (c) plasma sample from a patient who was receiving 20 mg of fluindione daily (3.0 μg/ml).

Table 1
Accuracy, precision, day-to-day and within-day reproducibility

Concentration added (μg/ml)	Concentration found: [mean C.V (%)], $n=10$	
	Day-to-day	Within-day
0.1	0.10 (6.1)	0.12 (1.6)
0.5	0.49 (2.7)	0.52 (2.8)
4	4.00 (0.3)	3.75 (2.3)

3.3. Plasma sample pretreatment and the I.S.

In spite of the fact that perchloric acid allows protein precipitation with a smaller dilution of plasma than acetonitrile, the procedure has not been chosen because it leads to degradation of fluindione (80%). To avoid the occurrence of peak broadening, it is strongly recommended to dilute the deproteinization supernatant 1:2 (v/v) (the concentration of acetonitrile is twofold higher in the deproteinization supernatant than in the mobile phase). Sodium chloride was used to bring the ionic strength close to that of the mobile phase. Phosphate buffer cannot be used because of the instability of fluindione and I.S. in this buffer.

Regardless of the level of plasma dilution, our quantification limit was found to be adequate for identification of steady-state levels of fluindione in blood [15].

Methods described for the quantification of other anticoagulants [9–14] required a large volume of plasma (1–2 ml) and a time-consuming extraction procedure. This is necessary to enhance the sensitivity and to avoid interferences with endogenous plasma compounds.

Roncato et al. [7] and Lotfi et al. [8] also used a large volume of plasma (1–2 ml) and an extraction phase to quantify fluindione. Despite this, their limit of quantification remains fourfold higher than ours (0.1 vs. 0.025 μg/l).

Although its retention time appears to be longer than that of fluindione, coumarin can be considered as a good I.S. as its λ_{max} (wavelength corresponding to maximum absorbance) is close to the wavelength used and it is stable in the solvents used. Furthermore, coumarin and fluindione, both exhibiting anticoagulant properties, should not be used simultaneously in patients and will not be present in the same blood sample.

The methods described for assaying fluindione [7,8] and other anticoagulants [9,11,12] do not usually involve the use of an I.S., even if there is a drug extraction step. However, for assaying coumarin, Lamiable et al. [14] use flunitrazepam as the I.S. As this drug is very commonly prescribed to patients in clinical practice, it makes it a source of analytical interference.

3.4. Stability

Spiked plasma samples, processed following the described procedure, remain stable at room temperature for at least 48 h, without significant degradation. Fluindione was stable in human plasma controls for more than six months at −22°C.

A stability study of fluindione in blood was performed under working laboratory conditions over a 120-h period, to check the stability of the molecule in the tube, once sampled. Tubes containing 5 ml of blood spiked with 0.1, 0.5 and 2.5 μg/ml of fluindione ($n=12$ at each concentration) were kept at room temperature (half were exposed to light). No differences (<5%) appeared between the concentrations found in blood samples exposed to light and those protected from light at three different levels of concentration. The plasma concentrations

Table 2
Stability of fluindione in spiked blood stored at room temperature at times 0, 72 and 120 h

Concentration added (μg/ml) in whole blood	Concentration found in plasma: (mean±SD) ($n=12$)		
	$t=0$ h	$t=72$ h	$t=120$ h
0.1	0.15±0.008	0.16±0.006	0.18±0.004
0.5	1.46±0.05	1.37±0.03	1.49±0.03
2.5	7.56±0.08	7.27±0.11	7.66±0.12

(mean±SD) of fluindione, assayed at times 0, 72 and 120 h, were similar for each concentration (Table 2).

Surprisingly, it appears that no proportionality exists between the amount of fluindione added to blood and the plasma concentration found after assay for the low level (0.1 μg/ml). This unexpected result might suggest a non-linear distribution of the drug in blood cells. Further in vitro studies could answer this question. Nevertheless, the results show that the fluindione remains very stable in blood after sampling. The stability of fluindione therefore allows samples to be sent by mail from peripheral clinical centers to the analytical unit.

4. Conclusion

This paper describes a novel method for the determination of fluindione in plasma. It is simple (no extraction procedure) and rapid (total time, 25 min). It requires only a 100-μl volume of plasma and is sensitive enough to perform drug monitoring of fluindione. Other antivitamin K drugs, such as indanedione (phenindione, diphacinone, chlorphacinone) and 4-hydroxycoumarin (acenocoumarol, ethyl biscoumacetate, brodifacoum, bromadiolone, coumatetralyl, difenacoum, warfarin) derivatives could also be assayed using this method, after modification of the proportion of acetonitrile in the mobile phase. However, these analyses would probably require an extraction step because of the low therapeutic levels of these drugs that are found in plasma from patients. This point is currently being investigated.

References

[1] E. Lefloch, M. Alhenc-Gelas, J.F. Paul, J. Emmerich, M. Aiach, J.N. Fiessinger, Presse Med. 24 (1995) 828.
[2] V. Cazaux, B. Gauthier, A. Elias, D. Lefebvre, J. Tredez, F. Nguyen, J.P. Cambus, B. Boneu, H. Boccalon, Thromb. Haemost. 75 (1996) 731.
[3] Y. Poinsignon, M. Hamidou, A. Chiffoleau, F. Banisadr, J.Y. Grolleau, C. Larousse, Rev. Med. Interne 15 (1994) 479.
[4] F. Grunenberger, L. Thiry, N. Sader, J.L. Schlienger, M. Imler, Rev. Med. Interne 16 (1995) 973.
[5] C. Andant, L. Aguettant, J.C. Soule, Gastroenterol. Clin. Biol. 21 (1997) 237.
[6] M. Porneuf, M. Dandurand, Int. J. Dermatol. 36 (1997) 138.
[7] M. Roncato, E. Billaud-Mesguich, J.N. Fiessinger, J.F. Vitoux, M. Aiach, J.M. Alexandre, Therapie 41 (1986) 203.
[8] H. Lotfi, M.F. Dreyfuss, P. Marquet, J. Debord, L. Merle, G. Lachatre, J. Anal. Toxicol. 20 (1996) 93.
[9] M.J. Fasco, L.J. Piper, L.S. Kaminsky, J. Chromatogr. 131 (1977) 365.
[10] F.A. de Wolff, C.A.M. Tetteroo-Tempelman, P.M. Edelbroek, J. Anal. Toxicol. 4 (1980) 156.
[11] T. Chalermchaikit, L.G. Felice, M.J. Murphy, J. Anal. Toxicol. 17 (1993) 56.
[12] E.A.P. Kuijpers, J. Den Hartigh, T.J.F. Savelkoul, F.A. de Wolff, J. Anal. Toxicol. 19 (1995) 557.
[13] D.A. Egan, R. O'Kennedy, J. Chromatogr. 582 (1992) 137.
[14] D. Lamiable, R. Vistelle, T. Trenque, R. Fay, H. Millart, H. Choisy, J. Chromatogr. 620 (1993) 273.
[15] F. Mentré, F. Pousset, E. Commets, B. Plaud, B. Diquet, G. Montalescot, A. Ankri, A. Mallet, P. Lechat, Clin. Pharmacol. Ther. 63 (1998) 1.