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Determination of the antifilarial drug UMF-078 and its metabolites UMF-060 and flubendazole in whole blood using high-performance liquid chromatography

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Abstract

A rapid and selective high-performance liquid chromatographic (HPLC) method for the simultaneous determination of the antifilarial drug UMF-078 (**I**) and its metabolites UMF-060 (**II**) and flubendazole (**III**) is described. After a simple extraction from whole blood, the compounds were determined by HPLC using a C_{18} Inertsil ODS-2 reversed-phase column with methanol–0.05*M* ammonium acetate (pH 4.0) as the mobile phase and ultraviolet detection at 291 nm. The average recoveries of **I**, **II** and **III** over the concentration range 20–500 ng ml^{-1} were 69.9 ± 4.7, 85.6 ± 4.4 and 85.1 ± 6.0%, respectively. The minimum detectable concentrations in whole blood for **I**, **II** and **III** were 10, 7 and 7 ng ml^{-1}, respectively. This method was found to be suitable for pharmacokinetic studies.

1. Introduction

Methyl (±)-[5-(α-amino-α-*p*-fluorophenyl)-1*H*-benzimidazol-2-yl]carbamate (UMF-078, **I**) (Fig. 1) is a broad spectrum anthelmintic agent. Like other imidazole derivatives, it is poorly absorbed from the gastrointestinal tract [1–3]. To be useful in the treatment of filariasis, it must be effective systematically and it is therefore desirable to determine its bioavailability. The exploration of these issues requires a sufficiently sensitive and reliable assay for **I** and its putative metabolites, methyl (±)-[5-(4-fluorophenyl)-hydroxymethyl-1*H*-benzimidazol-2yl] carbamate) (UMF-060, **II**) and methyl (±)-[5-(4-fluorobenzoyl]-1*H*-benzimidazol-2-yl]carbamate (flubendazole **III**). **I** [4] is an odourless white powder, having a melting point of 275–280°C with decomposition. It is very soluble in dimethyl sulphoxide (DMSO) but virtually insoluble in basic and neutral buffers. The drug has a first pK_a value of 3.8 and a second pK_a value of 8.0–8.5. **II** is a white powder which melts above 320°C. It is virtually insoluble in basic, acidic and neutral buffers, but very soluble in DMSO.

Flubendazole (**III**), one of the metabolites of **I**, has been determined by a radioimmunoassay in biological fluids [5]. However, this method, using antibodies, cannot discriminate between flubendazole and mebendazole. Further, in radioimmunoassay cross-reaction and length of the reaction limit its selectivity and sensitivity. A previously published method for the determination of UMF-058 [6] could not be used to

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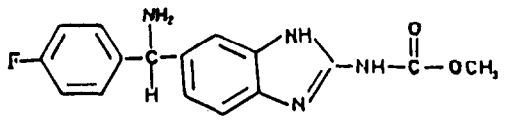
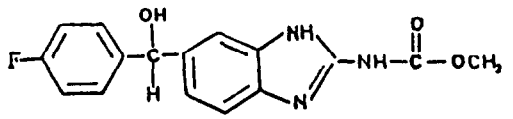


I



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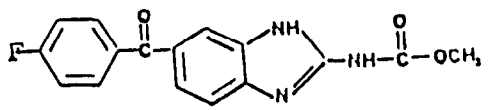

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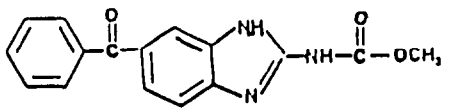

IV

Fig. 1. Structural formulae of I–IV.

determine **I**, which is a congener of UMF-058 but with different physico-chemical properties. **I** could only be extracted from a basic medium and requires a gradient chromatographic system for optimum separation. This paper describes a rapid, sensitive and selective HPLC procedure to determine **I**, **II** and **III** using mebendazole as an internal standard.

2. Experimental

2.1. Chemicals

I, **II**, **III** and mebendazole, [methyl (±)-(5-benzoyl-1*H*-benzimidazol-2-yl)carbamate] (**IV**) were obtained from Dr. L.B. Townsend (University of Michigan, Ann Arbor, MI, USA). All chemicals and solvents were of analytical-reagent grade. HPLC-grade methanol and ammonium acetate were purchased from Merck (Darmstadt, Germany). Diethyl ether was obtained from May and Baker (Dagenham, UK) and dimethyl sulphoxide and acetic acid from BDH (Poole, UK).

2.2. High-performance liquid chromatography

The HPLC system consisted of a Model 1051 binary pump (Waters, Milford, MA, USA), equipped with a Model 7125 syringe-loading sample injector with a 20-μl sample loop (Rheodyne, Cotati, CA, USA) coupled to a Lambda-Max Model 481 variable-wavelength ultraviolet absorbance detector (Waters) operating at 291 nm. Chromatograms were recorded using a Model 3392A electronic integrator (Hewlett-Packard, Avondale, PA, USA). Chromatographic separations were performed on a C_{18} reversed-phase stainless-steel column (150 × 4.6 mm I.D.; 5-μm particle size, Inertsil ODS-2) (GL Science, Tokyo, Japan) maintained at room temperature. The mobile phase was methanol (pump B)–0.05 *M* ammonium acetate (pump A) adjusted to pH 4.0 with acetic acid (17.5 *M*) at a flow-rate of 1 ml min^{-1}. Optimum separation of the four compounds of interest was achieved using the following gradient elution. Gradient elution commenced with pump A and pump B supplying 62% and 38% of the mobile phase components, respectively. At 6 min a gradient began that brought pump B to 55% at 9 min and then held these conditions up to 19 min. The gradient changed again at 19 min and returned to its initial condition (62% A, 38% B) at 21 min.

2.3. Extraction procedure

Extraction was carried out in 10-ml glass culture tubes silanized with dichlorodimethylsilane in toluene (5% v/v) in order to minimize adsorption on the glass. A 2-ml volume of boric acid–NaOH buffer (pH 10.5) were added to a sample of whole blood (1.0 ml) containing the internal standard IV (100 ng in 10 μl), **I**, **II** and **III** at various concentrations. The spiked blood sample was then vortex-mixed for 15 s. The mixture was extracted with diethyl ether (6 ml) by vortex-mixing for 1.5 min, followed by cen-

trifuging at 1000 *g* for 10 min. The supernatant (organic phase) was separated and evaporated to dryness under a gentle stream of nitrogen at room temperature (26°C). The residue was reconstituted in DMSO (100 μl) and 20 μl were injected for HPLC analysis.

2.4. Calibration

Stock solutions of **I**, **II**, **III** (2.5–10 ng μl^{-1}) and the internal standard **IV** (10 ng μl^{-1}) in DMSO were stored at 4°C. Calibration graphs were prepared by spiking drug-free whole blood samples with standard solutions (25–500 ng; 10–50 μl) of **I–IV** (100 ng; 10 μl) to give a concentration range of 25–500 ng ml^{-1}. The samples were taken through the extraction procedure described above and the peak heights of the drug and metabolites were plotted against their corresponding concentrations. Linear regressions of peak-height ratio *versus* drug or metabolite concentrations were performed in order to calculate the slope, intercept (peak-height ratio for zero concentration) and correlation coefficient for each calibration graph.

2.5. Analytical recovery and within-day and day-to-day precision

The absolute recoveries of the extraction procedures for **I–IV** were determined by comparing the peak heights obtained from extracted whole blood samples containing known amounts of the substance with those obtained from equivalent amounts of the compounds in DMSO by direct injection. Within-day and day-to-day precision were determined by replicate assays of samples from spiked whole blood. The day-to-day assay variation was assessed over a period of 5 days.

2.6. Animal study

A healthy monkey (*Macaca fascicularis*) received 129 mg kg^{-1} of **I** hydrochloride orally. Venous blood samples were taken before drug administration and at 0.5, 1, 2, 4, 6, 12, 36, 48, 72, 96, 120, 144 and 240 h afterwards. Blood was stored at −90°C until analysis by HPLC.

2.7. Pharmacokinetic analysis

Data are presented as means ± standard deviations. Elimination half-lives were calculated by regression analysis of the log-linear portion of the whole blood concentration *versus* time graph. The area under the whole blood concentration–time curve (AUC) was calculated by the linear trapezoidal rule. Other pharmacokinetic parameters (clearence and volume of distribution) were calculated using model-independent formulae [7]. Maximum concentration (C_{max}) and time to achieve the maximum concentration (T_{max}) were the observed values.

3. Results and discussion

The extraction procedure and chromatographic conditions yielded a clean chromatogram for **I**, **II** and **III**. Four peaks were resolved to the baseline with retention times of 9.1, 14.5, 17.3 and 19.3 min corresponding to **I**, **II**, **IV** and **III**, respectively (Fig. 2). Fig. 2 illustrates the chromatograms of (a) drug-free monkey (*Macaca fascicularis*) whole blood, and (b) whole blood of a healthy monkey following oral administration of **I** hydrochloride (129 mg kg^{-1}).

Mean recoveries from whole blood were 69.9 ± 4.7% (range 68.5–72.7%) for **I**, 85.6 ± 4.4% (range 78.0–92.3%) for **II**, 85.1 ± 6.0% (range 77.9–88.7%) for **III** and 82.1 ± 6.0% (range 75.6–85.4%) for **IV** (Table 1). Using 1 ml of whole blood, the minimum detectable concentrations of **I**, **II** and **III** were 10, 7 and 7 ng ml^{-1}, respectively. The within-day coefficients of variation (C.V.s) for **I**, **II** and **III** were in the ranges 3.9–5.8, 2.9–6.0 and 2.7–4.2%, respectively (Table 2). The day-to-day (five consecutive days) C.V.s for **I**, **II** and **III** were in the ranges 6.5–8.5, 7.0–7.3 and 4.5–5.1%, respectively (Table 3). The calibration graphs for **I**, **II** and **III** were all linear in the range 0–500 ng ml^{-1} ($r > 0.999$). The equations of the calibration graphs ($n = 6$) for **I**, **II** and **III** were $y = 0.005x + 0.02$, $y = 0.006x + 0.06$ and $y = 0.008x + 0.01$ respectively [y = peak-height ratio; x = concentration (ng ml^{-1})].

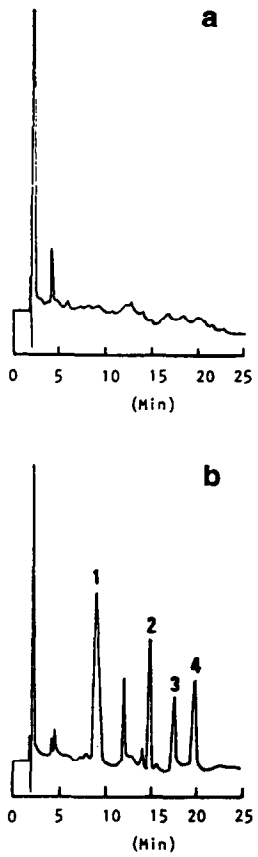

Fig. 2. (a) Chromatogram of drug-free healthy monkey (*Macaca fascicularis*) whole blood. Attentuation, 0.05 AUFS. (b) Chromatogram of whole blood of a healthy monkey at 96 h following the oral administration of **I** hydrochloride (129 mg kg^{-1}). Peaks: 1 = **I** (2455 ng ml^{-1}); 2 = **II** (434 ng ml^{-1}); 3 = internal standard (**IV**); 4 = **III** (267 ng ml^{-1}). Attenuation, 0.05 AUFS.

The validated method for the determination of **I**, **II** and **III** in whole blood was used to study their pharmacokinetics in a healthy monkey after a single oral dose (129 mg kg^{-1}) of the drug. The blood concentration–time profile over the period 0–240 h is shown in Fig. 3. The maximum blood concentration of **I** (11 952.7 ng ml^{-1}) was achieved at 6 h post-dose and the AUC was 747 161.1 ng ml^{-1} h. The clearence, volume of distribution and elimination half-life were 2.8 ml min^{-1} kg^{-1}, 10.1 l $kg^{-1}$and 40.5 h, respectively. **II** was found in blood 2 h post-dose with a maximum concentration (1074.7 ng ml^{-1}) at 72 h post-dose with AUC 67 143.5 ng ml^{-1} h. **III** could be detected in blood 2 h post-dose, with a maximum concentration (578.0 ng ml^{-1}) at 72 h post-dose, with AUC 39 752.4 ng ml^{-1}. The pharmacokinetic results show that the method was applied successfully for the simultaneous assay of **I**, **II** and **III** in whole blood.

The extraction procedure developed is facile as it requires a simple pH adjustment prior to extraction of these compounds. The moderate analysis time (20 min) together with rapid evaporation of diethyl ether (room temperature) allow

Table 1
Mean recoveries of **I**–**IV** ($n = 10$)

Compound	Spiked blood concentration (ng ml^{-1})	Mean recovery (%)	C.V. (%)
I	20	68.5	7.3
	100	68.5	8.2
	250	69.9	5.3
	500	72.7	4.6
II	20	84.9	7.0
	100	81.4	4.2
	250	88.6	4.7
	500	88.6	6.4
III	20	77.9	7.4
	100	88.7	4.7
	250	86.5	4.5
	500	86.8	5.4
IV	20	75.6	7.7
	100	82.8	4.3
	250	85.0	5.2
	500	85.4	5.4

Table 2
Within-day precision for assay of **I**–**III** ($n = 5$)

Compound	Spiked blood concentration (ng ml^{-1})	Concentration determined (ng ml^{-1})	C.V. (%)
I	50	45.9	4.6
	100	101.4	5.8
	250	265.3	3.9
II	50	54.7	4.0
	100	98.9	6.0
	250	254.4	2.9
III	50	52.4	4.2
	100	103.3	2.8
	250	251.5	2.7

Table 3
Day-to-day precision for assay of I–III ($n = 5$)

Compound	Spiked blood concentration (ng ml^{-1})	Concentration determined (ng ml^{-1})	C.V. (%)
I	50	49.8	8.3
	100	98.2	8.5
	250	243.9	6.5
II	50	50.4	7.3
	100	99.1	7.0
	250	251.4	7.1
III	50	50.1	4.8
	100	99.9	5.1
	250	245.5	4.5

rapid analyses, thus suitable for the routine analysis of large numbers of samples. However, it is important to note that this method is able to detect additional peaks, suggesting other possible metabolites of I or III, and work is in progress to characterize these peaks (Fig. 2b). In addition, this method works equally well with plasma and tissue homogenates. The within-day and day-to-day C.V.s were <10% in these samples.

In conclusion, the reported method for the determination of I, II and III satisfies all of the criteria required for an assay to be suitable for pharmacokinetic studies and has certain advantages, notably speed and low cost.

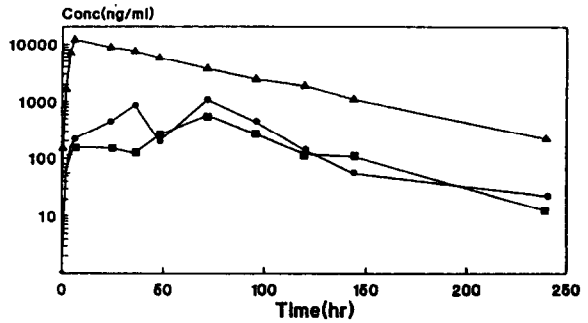

Fig. 3. Blood concentrations of (▲) I, (●) II and (■) III measured in healthy monkey (*Macaca fascicularis*) following the oral administration of a single dose (129 mg kg^{-1}) of I hydrochloride.

4. Acknowledgements

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