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Determination of the arotinoid mofarotene in human, rat and dog plasma by high-performance liquid chromatography with automated column switching and ultraviolet detection

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Abstract

A sensitive and specific high-performance liquid chromatographic method was developed and validated for the determination of the third-generation retinoid (arotinoid) mofarotene (Ro 40-8757) in human, rat and dog plasma, using direct injection of deproteinated plasma samples, automated column switching (on-line solid-phase extraction) and ultraviolet detection. Plasma (0.5 ml) was deproteinated by adding ethanol (1 ml) containing the internal standard Ro 42-8659 (200 ng/ml). After centrifugation, 0.9 ml of the supernatant were directly injected onto a precolumn packed with C_{18} Corasil 37–50 μm. Polar plasma components were washed out from the precolumn using 1% ammonium acetate–acetic acid–acetonitrile (900:9:100, v/v/v). After valve switching, the pre-concentrated compounds were transferred to the analytical column (C_{18}) in the backflush mode, separated by gradient elution and detected at 300 nm. The retention times (total run times) were approximately 15 and 20 min for the internal standard and mofarotene, respectively. The method was linear in the range 10–1000 ng/ml with a limit of quantification of 10 ng/ml. The mean recoveries were 80.4%, 81.7% and 77.8% (range 10–1000 ng/ml) and the inter-assay precision was 2.7% (range 20–1000 ng/ml), 1.5% and 2.0% (both range 100–1000 ng/ml) for human, rat and dog plasma, respectively. Mofarotene was found to be stable in human, rat and dog plasma stored at −20°C for 3 months and at 22°C for 24 h. The method was successfully applied to clinical, pharmacokinetic and toxicokinetic studies.

*Keywords:* Column switching; Mofarotene; Retinoids

1. Introduction

The arotinoid mofarotene (Ro 40-8757; 4-[2-(*p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)propenyl]phenoxy)ethyl]morpholine; Fig. 1) has been identified, so far, as the most effective member of the retinoid family in preclinical tumor models, particularly in chemically induced rat mammary carcinoma [1,2] and in human cancer cell lines

[3]. For its development as an anticancer compound, a rapid, sensitive and specific high-performance liquid chromatographic (HPLC) method was needed for the determination of mofarotene in rat, dog and human plasma samples from toxicokinetic and pharmacokinetic studies.

HPLC with ultraviolet (UV) detection is the method of choice for the determination of retinoids in biological samples [4,5]. HPLC with automated column switching (on-line solid-phase extraction) has been applied successfully to 13-*cis*- and all-*trans*-

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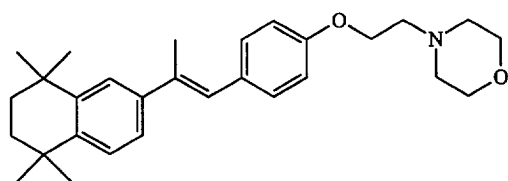
Mofarotene
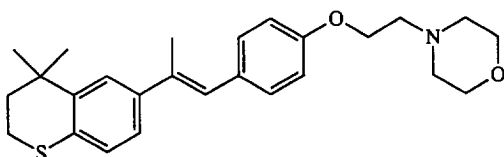
Ro 42-8659 (int. standard)

Fig. 1. Chemical structures of the compounds.

retinoic acid (isotretinoin and tretinoin) [6–11] and etretinate/acitretin [7,10,12,13] and their metabolites, as well as the arotinoid sumarotene [14].

This report describes the application of the column-switching technique to the determination of mofarotene, using a simple protein precipitation, reversed-phase HPLC and UV detection.

2. Experimental

2.1. Materials, reagents and solvents

Glacial acetic acid (100%) and ammonium acetate (both p.a.), and ethanol (absolute p.a. and HPLC grade), were obtained from E. Merck (Darmstadt, Germany), and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, UK). Water was distilled twice from an all-glass apparatus. Argon was obtained from PanGas (Lucerne, Switzerland). Mofarotene (Ro 40-8757/004) and Ro 42-8659/000 (I.S.) were provided by F. Hoffmann-La Roche Ltd (Basel, Switzerland) and were kept under argon at −20°C. Plasma standards were prepared using fresh frozen plasma either from sodium-citrated human blood, which was obtained from a blood bank (Blutspendezentrum SRK, Basel, Switzerland), or from rat or dog blood from our own laboratories

using potassium oxalate and EDTA as anticoagulants, respectively.

2.2. Preparation of standards

The preparation of plasma standards and the deproteination of the samples were performed under diffuse light conditions.

A stock solution of mofarotene was prepared in an amberized volumetric flask by dissolving 10 mg in 100 ml ethanol. Ultrasonication was used for complete dissolution. Appropriate amounts of the stock solution were diluted with ethanol to give working solutions in the range 100–1 μg/ml. These working solutions were used as plasma standards by diluting, e.g., 0.1 ml with blank plasma to 10 ml, yielding concentrations of 1000, 500, 100, 50, 20 and 10 ng/ml. The plasma standards were stored at −20°C.

A stock solution of the internal standard was prepared in an amberized volumetric flask by dissolving 10 mg of Ro 42-8659/000 in 100 ml ethanol (100 μg/ml). An internal standard working solution was prepared by diluting 0.2 ml of the stock solution with ethanol to 100 ml (200 ng/ml). This solution was freshly prepared prior to use. The stock solution could be stored at 4°C for several months.

2.3. Sample preparation procedure

A 1-ml aliquot of the ethanolic internal standard working solution was added to 0.5 ml plasma for protein precipitation. After vortex-mixing and standing for 15 min in the deep freezer at −20°C, the vial was centrifuged (6 min at 3400 g), the supernatant transferred to the autosampler vial (1.5 ml Safe-Lock, Eppendorf-Netheler-Hinz, Hamburg, Germany), and 0.9 ml were injected. Samples containing >1000 ng/ml of mofarotene were diluted by adding the supernatant to an adequate volume of internal standard working solution–water (2:1, v/v).

2.4. Chromatographic system and conditions

A schematic representation of the HPLC column-switching system is shown in Fig. 2. An HPLC-pump L-6000 (P1; Merck) in combination with a solvent selector (SS; Labsource, Reinach BL, Switzerland) delivered mobile phase M1 (or alternatively
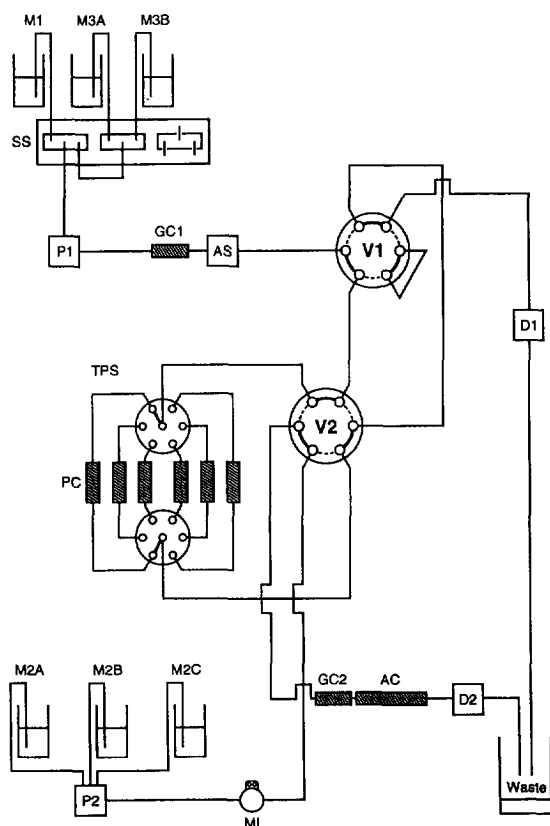
Fig. 2. Schematic representation of the HPLC column-switching system. Position of the valves: V1 = 10, V2 = 20 (step A). GC2 was not used in this method (see text for further details).

M3) at a flow-rate of 1.5 ml/min. Aliquots (0.9 ml) were injected by the autosampler (AS; Model AS-4000A, Merck) onto one of the precolumns (PC). In order to inject large sample volumes, the autosampler was used with two 5-ml syringes as diluters 1 and 2, a 2-ml sample loop, and the slow needle-down-speed. The UV detector D1 (Spectroflow 773, Kratos, Ramsey, NJ, USA), operating at 230 nm, together with a W+W recorder 600 (Kontron, Zurich, Switzerland), was used during method validation to monitor the removal of plasma components from the precolumn during the purge step; they were not needed for routine analysis. The gradient pump P2 (L-6200, Merck) delivered mobile phase M2 (flow-rate 1.0 ml/min), which was degassed on-line (Shodex DEGAS KT-35M degassing device, Showa Denko, Tokyo, Japan). A manual injector (MI; Model 7125 with a 200-μl loop, Rheodyne, Cotati,

CA, USA) was used for direct injection onto the analytical column (e.g. for recovery experiments). Detection of the eluted compounds was carried out at 300 nm with an UV detector (D2; Spectroflow 783, Kratos; rise time 1 s, range 0.01 AUFS), and integration was performed by a computing integrator (Model SP 4200, Spectra-Physics, San José, CA, USA; sensitivity 8 mV, chart speed 0.5 cm/min). For data-handling, a special BASIC program (similar to that described previously for the integrator SP 4100 [15]) was used with an in-house-developed laboratory information and management system (KIN-LIMS) [16].

The two electrically driven switching valves (V1 and V2; high-speed valve 7000E) and the solvent selector (all Labsource) were controlled by P2. Precolumns were automatically replaced by a tandem precolumn selector (TPS; EA6 Port valve 7066/CPR, Labsource), either when a pressure of 70 bar was reached, or after 100 injections.

Columns and mobile phases

The guard column GC1 and the precolumn PC (all 14×4.6 mm I.D., Bischoff-Analysentechnik) were packed with Bondapak C_{18} Corasil, 37–50 μm (Waters, Milford, MA, USA), and used with sieves (5 μm) without glass fibre filters to avoid column blocking. The analytical column (AC; 125×4 mm I.D., LiChroCART HPLC cartridge) contained Superspher 100 RP-18 (Merck) as stationary phase.

Mobile phase 1 (M1) was prepared by mixing 90 ml of 10% ammonium acetate with 810 ml of water, 100 ml of acetonitrile and 9 ml of acetic acid. Mobile phase 2 (M2) consisted of three components: (M2A) 8 ml of 10% ammonium acetate, 300 ml of water, 15 ml of acetic acid and 700 ml of acetonitrile, (M2B) 5 ml of 10% ammonium acetate, 20 ml of water, 10 ml of acetic acid and 980 ml of acetonitrile, (M2C) 10 ml of water, 5 ml of acetic acid and 990 ml of acetonitrile. Mobile phase 3A (M3A) and 3B (M3B) consisted of 80% and 100% acetonitrile, respectively.

Procedure

The total sequence of automated sample analysis required 27 min. Details of the parameters used are shown in Table 1. The autosampler started the integrator and the gradient program of P2. The timer

Table 1
Column-switching parameters and gradient program

Step	Time (min)	P2 gradient			P2 flow (ml/min)	P1 flow (ml/min)	P2 timer			Comment
		A (%)	B (%)	C (%)			V1	V2	SS	
A	0	100			0.05	0.05	10[a]	20		Injection of the sample onto PC.
	0.1				1.0	1.5			30	Polar components were washed
									40	out to waste.
B	7						11			PC was purged in the backflush mode.
C	8							21		Transfer of the retained
	8.2								31	components from PC to AC in the
	10								41	backflush mode. In the meantime,
	12							20		the capillaries between AS and D1
	15						10		40	were purged with M3.
	16	100							30	
	18.5		100							
	22				1.0					
D	24		100		3.0					Re-equilibration of PC with M1
	24.1			100						
	26.9			100	3.0					
	27	100			1.0	1.5				

[a] The first digit of the timer signal (1–4) represents the address and the second one (0 or 1) the activation.

signals of P2 were used for controlling the flow of P1, the switching valves (V1 and V2), the solvent selector, the pulse counter of the tandem precolumn selector and the start of the autosampler.

2.5. Calibration and calculations

Together with the unknown and quality control samples, six plasma standards, distributed over the whole set of samples, were processed as described above. The calibration curve ($y = a + bx$) was obtained by weighted linear least-squares regression (weighting factor $1/y^2$) of the measured peak-height ratios mofarotene/I.S. (y) versus the concentration of mofarotene (x).

3. Results and discussion

3.1. Sample preparation procedure

HPLC with column switching (on-line solid-phase extraction) was successfully applied to the determination of first-generation [6–11], second-generation [7,10,12,13] and third-generation [14] retinoids. The column-switching technique is especially suitable for retinoid determinations because of the sensitivity of these compounds to photoisomerization and oxidation. Two different methods of sample injection can be used to overcome low recoveries of the highly protein-bound retinoids which were obtained after injection of undiluted plasma samples [17]: (a) addition of a water-miscible organic solvent (e.g. acetonitrile) to the plasma sample and to M1 [6,7,9,10,13,14]; using a final proportion of less than 20% acetonitrile, plasma proteins are not precipitated, but the mass transfer of the retinoids from the protein to the stationary phase of the precolumn is improved; (b) protein precipitation with an organic solvent (e.g. ethanol) and injection of the supernatant [7,8,11,12]. The latter conditions, which are very robust and avoid any unintentional transfer of proteins to the analytical column, were used for mofarotene.

In contrast to our preliminary versions of the mofarotene method (R. Wyss and F. Bucheli, unpublished results), an ethanol–plasma ratio of 2:1 instead of 3:2 (to improve protein precipitation) and an injection volume of 0.9 ml instead of 0.5 or 0.6 ml (to improve the sensitivity) were used. Despite the high proportion of injected ethanol, no breakthrough of mofarotene appeared on the precolumn

owing to the interaction of the amine group with silanol groups of the stationary phase of the pre-column.

To reduce the number of sample-handling steps, the internal standard was added together with the ethanol for protein precipitation. No difference in recovery or precision could be observed when the internal standard was added in a smaller solvent volume prior to addition of the ethanol (data not shown).

3.2. Analytical system and chromatography

The advantages of the different valve-switching possibilities of the HPLC system were recently discussed for the arotinoid sumarotene [14]. A further improvement was realized by the incorpora-tion of a low-pressure three-way rotary valve and a mobile phase 3 for purging of the capillaries [13]. A solvent selector, as described in this report, sim-plified the system and allowed a choice between mobile phase M3A (80% acetonitrile) and M3B (100% acetonitrile). In this way, lipids could be eluted from the precolumn with M3B, preventing appearance of late running peaks in the following chromatograms. By introducing an intermediate purge step with M3A, precipitation of ammonium acetate from M1 in 100% acetonitrile was avoided. The precolumn was automatically replaced after 100 injections using the tandem precolumn selector.

Mofarotene is the first retinoid containing an amine group which has been analysed using the column-switching system for retinoids. Under the usual chromatographic conditions (Spherisorb ODS 1 as stationary phase, 0.04% ammonium acetate in mobile phase 2), mofarotene did not elute, due to interaction with silanol groups. The addition of an amine modifier (M2: (A) acetonitrile–water–hexyl methyl amine (850:150:0.5, v/v/v) and (B) aceto-nitrile–tetrahydrofuran–hexyl methyl amine (700:300:0.5, v/v/v)) allowed the determination of mofarotene. However, due to a baseline shift, the chromatographic system was far from ideal. There-fore, the stationary phase was replaced by Spherisorb Hexyl, which is known to be a well deactivated material for amines. As a result, tetrahydrofuran and hexyl methyl amine could be omitted from M2. However, the need to improve selectivity in human

plasma required further investigations. A Spherisorb ODS 1 column (again) in combination with a drasti-cally increased ammonium acetate concentration solved this problem. However, after several months of successful application of these conditions, the selectivity of the stationary phase deteriorated, prob-ably due to a change in the production process. Therefore, Spherisorb ODS 1 was finally replaced by Superspher 100 RP-18, which was found to have appropriate peak shape and selectivity.

After testing several candidates, Ro 42-8659, which was sufficiently separated from mofarotene and endogenous components, was chosen as internal standard. Typical chromatograms of a human blank plasma sample and a patient plasma sample taken after treatment with mofarotene are shown in Fig. 3 and Fig. 4, respectively. The retention times (total
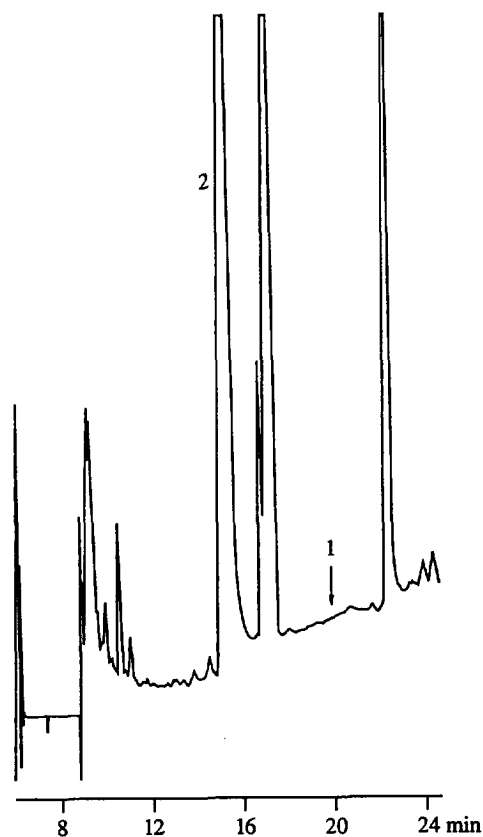

Fig. 3. Chromatogram of a human blank plasma sample. Peak identification: 1=mofarotene, 2=I.S. (Ro 42-8659).
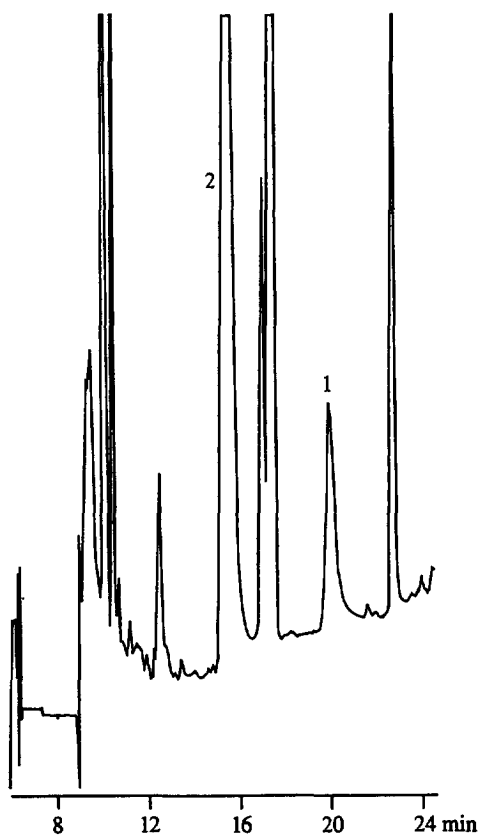
run times) were approximately 15 and 20 min for the internal standard and mofarotene, respectively. Although 285 nm is the absorption maximum of mofarotene, 300 nm was used in this method because of better selectivity.

3.3. Recovery

The recovery from plasma was determined during replicate analysis, by comparison of peak heights of spiked plasma samples, processed as described above, with 60-μl injections of standard solutions in mobile phase 2A. These solutions, directly injected onto the analytical column using MI, provided the 100% values. Under these conditions, acceptable recoveries were obtained (see Table 2).

3.4. Selectivity

In comparison to the preliminary methods, the actual conditions impart improved selectivity. More than ten different human blank plasma samples and blanks from several rats and dogs showed no endogenous interferences. Three derivatives of mofarotene without the morpholino group (structures not shown), which were tested as possible metabolites, did not interfere with mofarotene, nor did an in-vitro metabolite [18] which is eluted after about 12 min.

Fig. 4. Chromatogram of a patient plasma sample, taken 24 h after the last dose of a 45-day oral treatment of 50 mg/m^2. Measured concentration of mofarotene (1) 81.3 ng/ml. Peak 2 is the I.S. (Ro 42-8659).

Table 2
Recovery

Species	Compound	Concentration (ng/ml)		Recovery (%)	R.S.D. (%)	n
Man	Mofarotene	10		80.8	10.4	5
		100		80.1	3.8	5
		1000		80.3	1.4	5
	I.S.	400		82.4	2.2	15
Rat	Mofarotene	10		77.1	15.1	3
		100		83.4	3.1	3
		1000		84.7	0.3	3
	I.S.	400		81.3	2.9	9
Dog	Mofarotene	10		70.5	7.4	3
		100		83.1	3.4	3
			1000	79.8	1.8	3
	I.S.	400		79.9	2.6	9

3.5. Precision and accuracy

Inter-assay precision and accuracy were evaluated during method validation by analysing one series of plasma standards over six days against an independent calibration set. The results are compiled in Table 3.

3.6. Linearity

The method was linear in the range 10–1000 ng/ml, at least. Standard curves for mofarotene were calculated by means of weighted least-squares regression, using $1/y^2$ as weighting factor.

3.7. Limit of quantification

The limit of quantification was 10 ng/ml, using 0.5 ml of plasma. The inter-assay ($n=6$) relative standard deviations (R.S.D.) at this concentration were 9.6–13.7% for human, rat and dog plasma (see Table 3).

3.8. Stability

The stability of mofarotene was investigated by storing plasma samples spiked at different concentrations. The results of these stability tests, which were carried out according to our established method [19], are presented in Table 4. The data indicate that

mofarotene is stable in plasma of the investigated species. The small increase observed in some of the stability experiments was less than 5% and was, therefore, not considered to be relevant.

3.9. Application to biological samples

The method was successfully applied to clinical, pharmacokinetic and toxicokinetic studies. Fig. 3 and Fig. 4 show representative chromatograms from a clinical study. Overall, about 1500 plasma samples were analysed, demonstrating the robustness of the method.

4. Conclusions

An automated HPLC method was developed and validated for the determination of mofarotene in human, rat and dog plasma samples, using direct injection of deproteinated plasma samples and automated column switching. To improve the robustness of the method, protein precipitation was preferred to direct plasma injection. The chromatographic conditions were optimized for reducing peak tailing caused by interaction of the tertiary amine group of mofarotene with silanol groups of the stationary phase. The method was successfully applied to clinical, bioavailability and toxicokinetic studies.

Table 3
Inter-assay precision and accuracy from plasma standards during method development ($n = 6$)

Species	Concentration (ng/ml)		R. S. D. (%)	Difference between found and added (%)
	Added	Found		
Man	10	9.89	11.7	−1.1
	20	19.8	8.7	−1.0
	50	52.1	1.7	+4.2
	100	99.0	0.7	−1.0
	500	514	1.2	+2.8
	1000	973	1.1	−2.7
Rat	10	9.70	13.7	−3.0
	100	99.1	0.9	−0.9
	1000	977	2.1	−2.3
Dog	10	9.63	9.6	−3.7
	100	102	2.1	+2.0
	1000	970	1.9	−3.0

Table 4
Stability of mofarotene in plasma

Species	Storage conditions	n	Concentration (ng/ml)	Change in conc. after storage (%)	90% Confidence interval (%)
Man	24 h at 22°C	5	50	−3.1	−5.2 to −0.9
		5	500	−1.3	−2.1 to −0.4
		5	10000	−3.3	−6.3 to −0.3
	3 months at −20°C	5	50	+5.0	+3.0 to +7.1
		5	500	−1.7	−2.2 to −1.2
		5	10000	−6.8	−8.1 to −5.4
Rat	4 h at 22°C	3	500	+3.2	−0.6 to +7.2
	24 h at 22°C	3	500	+3.7	+1.3 to +6.2
	1.5 months at −20°C	4	500	+1.3	+0.5 to +2.1
	3 months at −20°C	5	500	+3.8	+3.2 to +4.3
Dog	4 h at 22°C	3	500	+3.0	−0.3 to +6.3
	24 h at 22°C	3	500	+2.9	+0.2 to +5.6
	1.5 months at −20°C	5	500	−0.4	−1.5 to +0.7
	3 months at −20°C	5	500	+2.6	+1.5 to +3.7

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