Determination of Adapalene (CD271/Differin®) and Retinol in Plasma and Tissue by On-Line Solid-Phase Extraction and HPLC Analysis

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Key Words

Column liquid chromatography
Solid-phase extraction
Differin
Retinol
Adapalene

Summary

Adapalene, the active constituent of Differin®, is a novel potent retinoid (vitamin A analogue) for the topical treatment of acne vulgaris. The clinical usefulness of retinoids is limited by a number of side effects, such as teratogenicity and skin irritation. A method has been developed for simultaneous determination of adapalene and retinol in plasma and tissue in in vivo and in vitro studies for the determination of the pharmacokinetic profile and the influence of adapalene on the endogenous retinol level. The new method was developed by coupling an autosampler to an automated solid-phase extraction unit on-line with a gradient HPLC system using UV and fluorescence detection. The low detection limit (0.25 ng mL^{-1} for adapalene), the small sample weight (50 mg) and the high degree of automation make this method convenient for analysis of biological samples in animal and human studies.

Presented at the 21st ISC held in Stuttgart, Germany, 15th–20th September, 1996

Introduction

Retinoids, derivatives of vitamin A alcohol (retinol), are involved in several physiological processes such as embryonic development, growth and differentiation [1, 2].

In therapy retinoids are used in the treatment of acne vulgaris, photoaged skin and hyperkeratotic skin disorders [3]. The clinical usefulness of retinoids is limited by a number of side effects such as skin irritation and teratogenicity [4–6]. Adapalene (Differin®), a new synthetic retinoid of the naphthoic acid series (Figure 1), was developed for the topical treatment of acne vulgaris; in comparison with naturally occurring retinoids it is chemically and photochemically stable [7]. In terms of pharmacology, adapalene behaves similarly to the well known commercially available retinoid tretinoin [8]. In clinical studies the efficacy of adapalene was comparable with, if not superior to, that of tretinoin, but adapalene was better tolerated. In comparison with tretinoin gel, adapalene gel induced less erythema, dryness, scaling and burning after application [9].

Plasma and tissue levels of adapalene are important for the estimation of the teratogenic risk of the topically administered adapalene. In cutaneous retinoid therapy, systemic exposure is low [10].

We have developed a fully automated reversed-phase HPLC method for the parallel determination of adapalene and retinol using automated on-line solid-phase extraction. The method has been used for the determination of the concentration of the retinoids in human plasma and in mouse kidney and embryo. The new method can also be used in pharmacokinetic studies for evaluation of placental transfer and the distribution of adapalene and its metabolites in various maternal tissues. Several metabolites could also be separated with this method [11].

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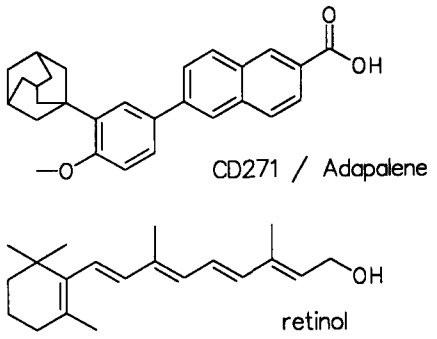

Figure 1
Chemical structures of CD271 (Adapalene) and retinol.

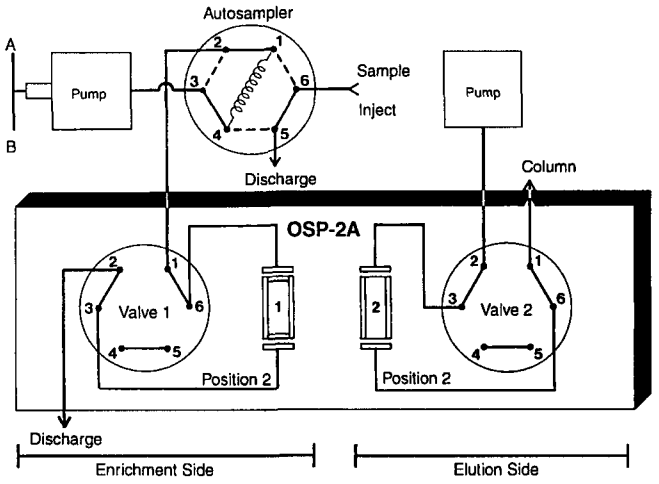

Figure 2
Schematic diagram of the OSP-2A solid-phase extraction unit from the analytical system.

Experimental

Chemicals

Adapalene (CD271; 6-(3-(1-adamantyl)-4-methoxyphenyl)-2-naphthoic acid) was kindly provided by CIRD-Galderma [12] (Sophia Antipolis, France), retinol was obtained from Serva (Heidelberg, Germany) and bovine serum albumin (BSA) was obtained from Sigma (Deisenhofen, Germany). Methanol, isopropanol, acetonitrile and ammonium acetate were purchased from E. Merck (Darmstadt, Germany). Water was deionized and purified by means of a Milli-Q system (Millipore, Eschborn, Germany).

Laboratory Precautions

As retinol is sensitive to light, all experiments (animal treatment and analytical work) were performed under dim yellow light.

Sample Collection for Measurement of the Pharmacokinetics of Adapalene

Mice (NMRI) of gestation day (GD) 11 were treated with CD271 (21 mg kg^{-1} body weight) by subcutaneous application of a DMSO solution (1 mL kg^{-1}). Treated animals were killed by decapitation after selected time intervals. Plasma, embryo, liver and kidney were collected and stored at -20 °C before analysis. Maternal plasma was collected into heparinized funnels and heparinized 1-mL tubes. Plasma was prepared by centrifugation (10 min at 1500 g and 4 °C). Embryo and maternal tissues were quickly removed [11].

Sample Pretreatment

Plasma (ca. 80 µL) was accurately diluted with a 3-fold excess of acetonitrile. After 3 min shaking the precipitated protein was pelleted by centrifugation. Embryo samples were diluted with a 3-fold volume of isopropanol and disrupted by ultrasonic treatment on ice (B 12 sonicator, setting 2.5; Branson Sonic Power Company, Danbury, CT, USA) before shaking. Kidneys from one animal were pooled, diluted with 1 volume of water, minced with scissors, and 80 µL of this preparation was diluted with a 3-fold excess of isopropanol and disrupted by ultrasonic treatment on ice. Liver was diluted with a 9-fold excess of ice-cold 0.9 % aqueous NaCl solution, homogenized with a Teflon-glass potter, and 80 µL of the homogenate was diluted with a 3-fold excess of isopropanol and disrupted by ultrasonic treatment on ice [13].

After the treatment described above the sample was centrifuged for 5 min and the supernatant (ca. 260 µL) was accurately diluted with one volume of 0.5 % aqueous ammonium acetate solution and placed into the AS-4000 autosampler (as described below) [14].

Solid-Phase Extraction

Sample enrichment and clean-up were performed by use of an on-line solid-phase extraction unit, consisting of an L-6200A intelligent pump and an intelligent AS-4000 autosampler (both from E. Merck, Darmstadt, Germany) with a M4000TH sample thermostat (4 °C; Cheminst, Berlin, Germany). The autosampler was connected to an OSP-2A programmable on-line solid-phase extraction unit (E. Merck; Figure 2) that performed fully automated sample enrichment and clean-up and incorporation of the loaded cartridge (OSP-2A cartridges, LiChrospher® 60, RP-18, 10 µm; E. Merck), by a valve-switching technique, into the analytical cycle.

Before sample application the cartridges were pre-conditioned and equilibrated. The biological extract (400 µL) was then automatically injected by the AS-4000 program (Table I) by valve-switching into the

Table I. Program of the AS-4000 autoinjector.

1. DO 70 1	Start of the program 1 max. 70 times.
2. WASH	Wash the needle twice with the purge solution (2 % aqueous ammonium acetate solution).
3. VALVE 1	Valve of the AS-4000 to the loading position.
4. TUBE 2I1	Take the needle to the tube from the rack 2 (M4000TH) at position 1 and with a step rate 1 for the following position at the next cycle.
5. ASP 400 1	Aspirate 400 µL of the solution in the tube, with AS-4000 speed code 1, with the 0.5-mL syringe and 0.5-mL sample coil.
6. TUBE 0 1	Needle to the injection port of the AS-4000.
7. DISP 450 1	Dispense 450 µL of the solution in the needle and sample coil into the sample loop (500 µL).
8. VALVE 0	Valve of the AS-4000 to the injection position.
9. OUT 1.1	
10. WAIT 1.0	
11. OUT 1.0	9.–11. Starting signal for the L-6200A program.
12. WAIT 1200.0	Wait for 1200 s (20 min) for the next cycle.
13. LOOP	Go to step No 1.

Table II. Composition of the multi-linear binary gradient and program for automatic (on-line) sample preparation and injection of the L-6200A for the OSP-2A.

Time (min)	Solvent A[a] %	Solvent B[b] %	Flow (mL min^{-1})	Event	Orders and remarks
0.0	15	85	0		Sample injection by AS-4000 and start of the program.
				11	Valve 1 to the loading position 1.
				31	Close the clamp.
0.1	15	85	1		
2.1	15	85	1		
2.2	100	0	0		
2.3				30	Open the clamp.
2.4				42	Transport the cartridge one step to the analytical cycle.
2.5	100	0	1	31	Close the clamp.
2.6	100	0	1	21	Valve 2 to the eluting position.
2.7	100	0	1	72	Start signals for the integrator and
				82	the HPLC controller.
2.8	100	0	3	11	Valve 1 to the loading position.
5.8	100	0	3		
5.9	0	100	0		
6.0	0	100	0.5		
11.0	0	100	0.5		
16.5	0	100	0	30	Open the clamp.

[a]Acetonitrile. [b]2 % Aqueous ammonium acetate solution (pH 4).

sample enrichment cycle; the time program of the L-6200A was simultaneously started by the AS-4000 and the sample was eluted by means of a multi-linear binary gradient. The timed events for the multi-linear gradient and the OSP-2A functions (Table II) were defined in the time program of the L-6200A. The chromatographic analysis was also started by an impulse from the time program of the L-6200A, at which point elution of the cartridge in the backflush mode was initiated. During the analysis the next cartridge was cleaned, preconditioned and loaded [14].

Chromatographic System

The HPLC system consisted of two LC-10AD HPLC pumps connected to an SCL-10A system controller including a optical PC-interface and Class-software as controller and integrator. A dynamic mixing chamber (Mixer SUS; P/N 228-28000-91), a two-channel SPD-10AV UV/Vis detector and a RF-530 fluorescence monitor (all from Shimadzu, Duisburg, Germany) were also components of the analytical equipment. The eluents were degassed using a Degasys DG-1310 (VDS

Optilab, Berlin, Germany) before mixing, then passed through an in-line filter (1–2 µm; Knauer, Berlin, Germany) before reaching the analytical column (120 × 4 mm i.d.), packed in our laboratory with 3 µm Spherisorb ODS2 (Phase Separations, Deeside, UK), embedded in a 7970 Jones Chromatography block heater (VDS Optilab, Berlin, Germany) in which the column was heated to 60 °C. A multi-linear gradient was produced from solvent A (1:1, *v/v*, 0.5 % aqueous ammonium acetate solution (pH 4) – methanol) and solvent B (1:1, *v/v*, methanol – isopropanol); the composition of the gradient is given in Table III. The flow rate was 0.7 mL min^{-1}. The detection wavelength was 320 nm at the UV detector and 437 nm emission and 320 nm excitation at the fluorescence monitor.

Standard Solutions

Stock solutions were prepared by dissolving the two retinoids (10 mg) in ethanol (100 mL), to give a final concentration of 100 µg mL^{-1}. All stock solutions were stored at –20 °C.

Calibration

The reference retinoid solutions used for validation of the assay were all prepared by spiking a solution of bovine serum albumin (BSA) in phosphate-buffered saline, human plasma, mouse embryo extract and mouse kidney extract with ethanolic solutions of the retinoids (CD271 and retinol). Multi-linear calibration was performed by measurement of three standards (10, 100, 1000 ng mL^{-1}) of the two retinoids. Calculation of response factors and coefficients of correlation was performed automatically by the Class software. The correlation coefficients, *r*, for the linearity of the plots (2.5, 5, 10, 50, 100, 500, 1000, 2000 ng mL^{-1}) were always greater than 0.999. The concentration of retinol was determined by UV detection and the concentration of CD271 by the fluorescence monitor.

Table III. Composition of the mobile phase (multi-linear binary gradient).

Time (min)	% Solvent A[a)]	Solvent B[b)]
0.0	90	10
8.0	10	90
8.5	10	90
17.0	5	95
17.4	5	95
17.5	90	10

[a)] 1:1, *v/v*, 0.5 % aqueous ammonium acetate solution (pH 4) – methanol
[b)] 1:1, *v/v*, methanol – isopropanol.

Results

Recovery, Precision and Limit of Detection

Recovery was determined by comparing the peak areas obtained from spiked samples (n = 6) with those obtained by direct manual injection (n = 6) of three different concentrations (10, 100, 1000 ng mL^{-1}). The recoveries from BSA solution, human plasma, mouse embryo extract and mouse kidney extract were > 71 %, > 74 %, > 71 % and > 68 %, respectively (Table IV).

Intra-day variation (relative standard deviation, *RSD*) was usually < 7.4 % (n = 6), and the inter-day variation from three consecutive days was < 10.2 % (n = 3) (Table V).

As little as 0.25 ng mL^{-1} CD271 and 2.5 ng mL^{-1} retinol could be detected in a 50 µL (50 mg) sample of human plasma.

Experimental Application

Figure 3 shows typical chromatograms obtained after extraction of BSA and mouse kidney extracts spiked with standard solutions of adapalene and retinol.

Discussion

This HPLC method enables quantitative determination of adapalene (CD271), the active constituent of Differin, and retinol in human plasma and mouse tissues in a single-run separation of both retinoids. The method is automated to a very large extent. The sample prepara-

Table IV. Recovery (compared with direct injection of the respective amount of retinoids) of CD271 from BSA solution, human plasma, mouse embryo extract and mouse kidney extract, and of retinol from BSA solution.

Retinoid	Recovery (%)		
	10 ng mL^{-1}	100 ng mL^{-1}	1000 ng mL^{-1}
CD271 in BSA solution	77 ± 5	71 ± 1	77 ± 4
CD271 in human plasma	82 ± 3	86 ± 2	91 ± 3
CD271 in mouse embryo extract	83 ± 4	82 ± 2	89 ± 2
CD271 in mouse kidney extract	82 ± 4	99 ± 4	89 ± 1
Retinol	71 ± 4	76 ± 1	83 ± 6

Values are based on the main peak area values of six standard samples and six direct injections.

Table V. Intra-day and inter-day reproducibility expressed as *RSD*.

	Intra-day *RSD* (%; $n = 6$)			Inter-day *RSD* (%; $n = 3$)		
	10 ng mL^{-1}	100 ng mL^{-1}	1000 ng mL^{-1}	10 ng mL^{-1}	100 ng mL^{-1}	1000 ng mL^{-1}
CD271	4.5	1.9	6.0	3.6	4.3	5.8
Retinol	3.4	1.9	7.4	10.2	4.9	7.8

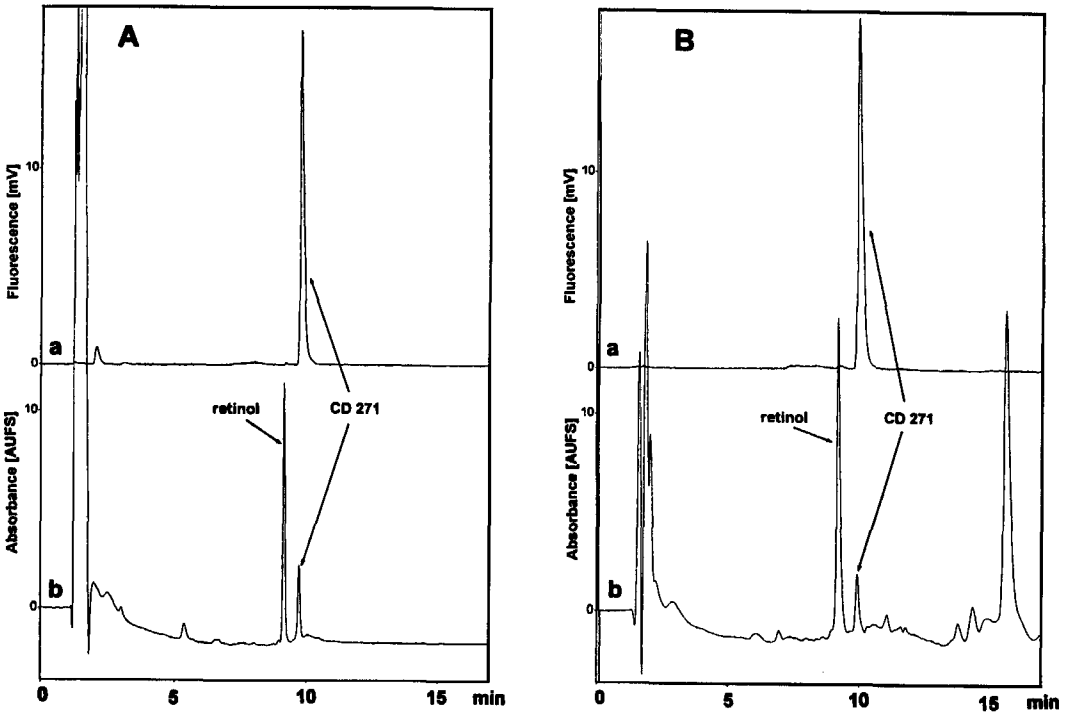

Figure 3
Chromatograms of CD271 (adapalene) and retinol in BSA solution (A) and in the kidney extract (B) of an untreated pregnant mouse (GD 11). BSA solution and mouse kidney extract were spiked with each retinoid (100 ng mL^{-1}). Detection wavelengths were 437 nm emission and 320 nm excitation at the fluorescence detector (chromatogram a) and 320 nm at the UV detector (chromatogram b).

tion system including the AS4000 autosampler and the OSP-2A solid-phase extraction system is reliable and provides a range of additional functions. The sample pretreatment step is kept simple by diluting the sample with a three-fold volume of an organic solvent and centrifugation, followed by direct injection of the supernatant into the HPLC system. This automation ensures that all samples are processed in the same way and that time-consuming sample preparation can be reduced to a minimum.

The assay sensitivity of the method was 0.25 ng mL^{-1} (ng g^{-1}) for adapalene and 2.5 ng mL^{-1} for retinol in a 50-mL (50-mg) sample. The low detection limit is of particular importance for adapalene because this compound is active at very low concentrations.

In routine analysis the separation and detection of still unknown adapalene metabolites is also possible. Figure 4 shows chromatograms obtained from plasma and kidney samples obtained from a pregnant mouse. The method has been used successfully in a pharmacokinetic study of adapalene [11].

The method is linear in the range 2.5–2000 ng mL^{-1} (ng g^{-1}) for the two retinoids ($r > 0.999$).

High sensitivity, a high degree of automation and high reproducibility make this method convenient and reliable for use in animal and human studies.

Acknowledgements

We thank Dr. Reichert and Dr. Shroot (CIRD Galderma, Sophia Antipolis, France) for support and for the gift of adapalene. We thank Dr. Karbus (E. Merck, Darmstadt, Germany) for helpful suggestions and for his support with the instrumentation. We are also grateful to Dr. R. Thiel for conducting the animal experiment.

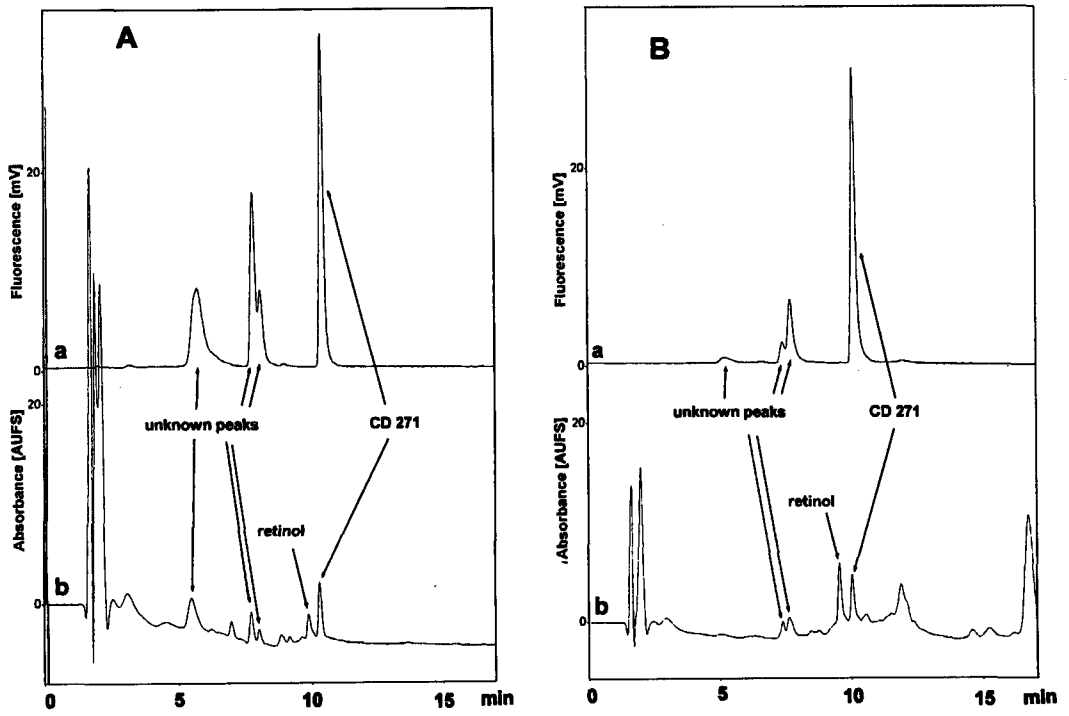

Figure 4
Chromatograms of plasma (A) and kidney samples (B), obtained from a pregnant mouse (GD 11) 6 h after subcutaneous treatment with CD271 (adapalene; 21 mg kg^{-1} body mass). Detection wavelengths were 437 nm emission and 320 nm excitation at the fluorescence detector (chromatogram a) and 320 nm at the UV detector (chromatogram b).

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Received: Sep 16, 1996
Accepted: Dec 2, 1996