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Synthesis, high-performance liquid chromatography–nuclear magnetic resonance characterization and pharmacokinetics in mice of CD271 glucuronide

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Abstract

Retinoic acid-glucuronides are known as retinoids with activity in acne therapy, limited placental transfer and reduced retinoid adverse effects. We synthesized the glucuronide of a novel retinoid, CD271 (adapalene), used for the treatment of moderate acne. The synthesis product (''CD271 glucuronide'', CD271G) was purified by preparative HPLC. It undergoes in aqueous solution, like other glucuronides, rapid acyl-migration of the bound aglycone leading to position isomers. Thus characterization of purified CD271G could be only achieved by HPLC–NMR coupling. A subfraction (''CD271GB'') consisting essentially of 2′- and 3′-CD271G was used for pharmacokinetic studies. After a single subcutaneous injection at a dosage of 30 mg/kg the substance showed considerable uptake and metabolism to CD271 indicating that CD271GB could serve as a prodrug for CD271.

Keywords: CD271 glucuronide

1. Introduction

CD271 (adapalene, Differin) is a new synthetic retinoid of the naphthoic acid series, and was developed for the topical treatment of Acne vulgaris; in contrast to the naturally occurring retinoids it is photochemically and chemically stable [1]. In clinical studies the efficacy of CD271 was comparable with, if not superior to that of tretinoin (all-*trans*-retinoic acid, ATRA), but CD271 was better tolerated. In comparison with tretinoin gel, adapalene gel induced less erythema, dryness, scaling and burning after application [2].

The glucuronide of all-*trans*-retinoic acid (ATRAG) also shows beneficial effects on acne without any teratogenic effects [3]. It exerts retinoid-

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like biological activity in a variety of in vitro systems such as cultured human promyelocytic leukemia cells [4,5] and mammary glands in organ culture [6]. Analytical measurements revealed that a considerable portion of ATRAG is hydrolyzed in vitro to the resulting aglycone ATRA, which was found in the medium as well as in the cultured tissue [7–9]. Nau et al. [10] demonstrated immediate hydrolysis of ATRAG to ATRA after subcutaneous and intravenous administration to mice. Endogenous ATRAG has been detected as a single positional isomer of the retinoic acid-glucuronide without any detection or identification of positional isomers. The stability of ATRAG against rearrangement could also be the reason of better cleavage by specific glucuronidases and considerable hydrolyzes to the aglycone. Retinoic acid-glucuronide does not interact with cytosolic retinoic acid binding proteins [11] or retinoic acid receptors [12] which favors the hypothesis of an indirect action of these glycoconjugates.

Several glucuronides undergo rearrangement of the aglycone bound to a specific position of the glucuronic acid via acyl migration [13–16]. Acyl migration is a fast process in aqueous solution and results rapidly in a mixture of positional isomers of the glucuronide. It is evident that the time consuming sample preparation after conventional HPLC separation, which could lead to an undefined mixture of positional isomers [17] and the aglycone [18,19], is not suitable for such a situation, which becomes even more complicated by the formation of covalent adducts with low-molecular-mass nucleophiles (such as methanol) and proteins [20,21]. Thus coupling of high-performance liquid chromatography (HPLC) with nuclear magnetic resonance (NMR) spectrometry, in which the NMR instrument is equipped with a flow cell and the usage of an acidic solvent, is certainly the more appropriate analytical tool. This technique has also been used for the on-line detection of vitamin A derivatives [22–24].

Biological activity and the low placental transfer of retinoic acid-glucuronides prompted us to synthesize a novel retinoid-glucuronide with a lower susceptibility to cleavage by endogenous glucuronidases. In the following we describe the synthesis of CD271 glucuronides and study the pharmacokinetic properties of the subfraction CD271GB, consisting essentially of 2′- and 3′-CD271G, obtained after preparative HPLC purification. CD271G should be used as a prodrug with superior pharmacokinetic properties in comparison to the aglycone and therefore the in vivo experiments have been performed with easy accessible CD271GB.

Identification of the used positional isomers for in vivo experiments and pharmacokinetic studies of CD271GB, as a putative drug for dermatological usage, to CD271 as the active substance has been the major aim of the following experiments.

2. Experimental

2.1. Chemicals

All solvents for the HPLC separation were purchased from Merck KGaA (Darmstadt, Germany), all chemicals for the synthesis were from Sigma (Deisenhofen, Germany). CD271 was provided by Galderma R&D (Sophia Antipolis, France). Water was deionized and purified by means of a Milli-Q system (Millipore, Eschborn, Germany).

2.2. Synthesis of CD271-glucuronide

CD271 was solubilized in methylene chloride and fluorinated using diethylaminosulfurtrifluoride (DAST) as the fluorinating agent (Fig. 1) as

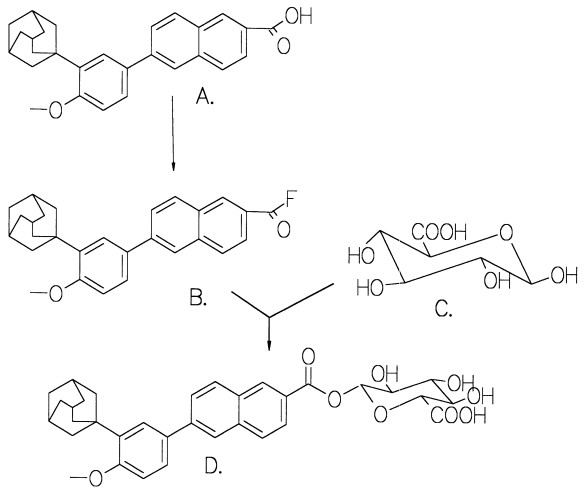

Fig. 1. Reaction scheme for the synthesis of CD271-glucuronide (D). CD271 (A), CD271-fluoride (B), glucuronic acid (C).

described for the synthesis of all-*trans*-retinoyl-fluoride [25]. The product (CD271F) was a white solid with a melting point of 232–233°C.

^{1}H-NMR (dimethyl sulfoxide, DMSO): δ 1.9 (s, 6H, CH_2-adamantyl); 2,0 (s, 3H, CH-adamantyl); 2,1 (s, 6H, CH_2-adamantyl); 3,9 (s, 3H, $-OCH_3$); 7,0 (d, 1H, J=7.8 Hz, 5-phenyl); 7.2 (s, 1H, 2-phenyl); 7.6 (dd, 1H, J=7.8 Hz, 6-phenyl), 7, (dd, 1H, J=7.8 Hz, 7-naphthyl); 8.0 (m, 1H, 4-naphthyl); 8.1 (d, 1H, J=7.8 Hz, 8-naphthyl); 8.2 (dd, 1H, J=7.8/3.6 Hz, 3-naphthyl); 8.2 (s, 1H, 5-naphthyl); 8.6 (s, 1H, 1-naphthyl) ppm.

A 1-g (2.41 mmol) amount of CD271F was solubilized in 1000 ml acetone to which a solution of 2.5 g (12.9 mmol) glucuronic acid and 2.5 g sodium hydrogencarbonate in 1000 ml water was added. The mixture was stirred at 40°C for 72 h. After addition of 500 ml water stirring was continued for another 24 h. The reaction mixture was acidified with 6 *M* HCl until a pH of 6 was obtained. The solution was extracted two times with ethyl acetate and methylene chloride until the solution became colorless under UV light (254 nm). The organic phase was distilled and the resulting liquid was purified on a silica gel column using hexane–ethyl acetate (1:1) as the solvent. The substance was eluted from the column with methanol. The solvent was distilled off in a rotary evaporator. A white solid substance (0.82 g) was obtained. The compound was purified by reversed-phase HPLC (Eurosil Bioselect 100-20 C_{18} 250×32 mm column with a 40×32 mm pre-column) using a Shimadzu SPD-6A UV detector, a preparative flow cell (3NL) and a Knauer HPLC pump with a preparative pumphead. The solvent was 2% (w/v) aqueous ammonium acetate (pH 7.4) solution–methanol (22.5:77.5). The flow-rate was 25 ml/min. Purified CD271G was obtained after 40 min (Fig. 2). The methanol of the peak eluate was evaporated and the yielded aqueous phase was acidified with HCl and extracted several times with ethyl acetate. The ethyl acetate extract was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness in a rotary evaporator. The structural composition of CD271Gs after synthesis was determined by HPLC–NMR coupling (Fig. 3). A subfraction (''CD271GB'') obtained by preparative HPLC and consisting mainly of 2′- and 3′-CD271G was used for the pharmacokinetic experiments.

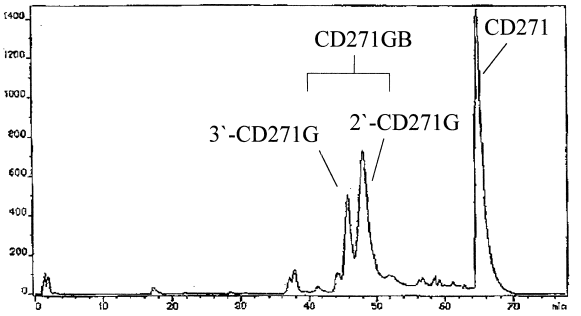

Fig. 2. Chromatogram after HPLC separation of CD271G on an Spherisorb ODS 2A 3 μm column. The gradient was formed by two solvents: (A) deuterium oxide; (B) acetonitrile; at a column temperature of 10°C and UV detection at 326 nm. The following gradient was used: 0 min 40% B; 40 min 55% B; 48 min 55% B; 55 min 90% B; 60 min 90% B; 65 min 40% B at a flow of 0.7 ml/min.

2.3. HPLC–NMR measurements

The HPLC system consisted of a Merck–Hitachi L6200A HPLC pump, a Merck–Hitachi L4000A UV detector (both from Merck KGaA) and Bruker peak sampling unit BPSU-12 from Bruker (Rheinstetten, Germany). The system was controlled by Bruker LC-Chromstar software and the Bruker LC244M interface. The outlet of the BPSU was connected via a 3 m polyether ether ketone (PEEK) capillary (0.25 mm I.D.) to a 200 μl flow probe with a radio-frequency coil arranged for inverse $^{13}C/^1H$ spectroscopy. The spectrometer was a Bruker ARX 400 (9.4 T) equipped with Bruker UXNMR software. The separation was performed on a 200×4 mm HPLC column (Spherisorb ODS 2A) using a gradient of deuterium oxide as solvent A and acetonitrile with 0.2% trifluoroacetic acid as solvent B. The applied gradient was 0 min 40% B, 40 min 55% B, 48 min 55% B, 55 min 90% B, 60 min 90% B and 65 min 40% B at a flow-rate of 0.7 ml/min. The detector wavelength was set to 320 nm.

2.4. Animals and treatment

NMRI mice (Han: NMRI; Zentralinstitut für Versuchstierkunde, Hannover, Germany) were kept under specific pathogen free (spf) conditions and a 12-h standard light–dark cycle at 21±1°C and 50±5% relative humidity. They received a standard

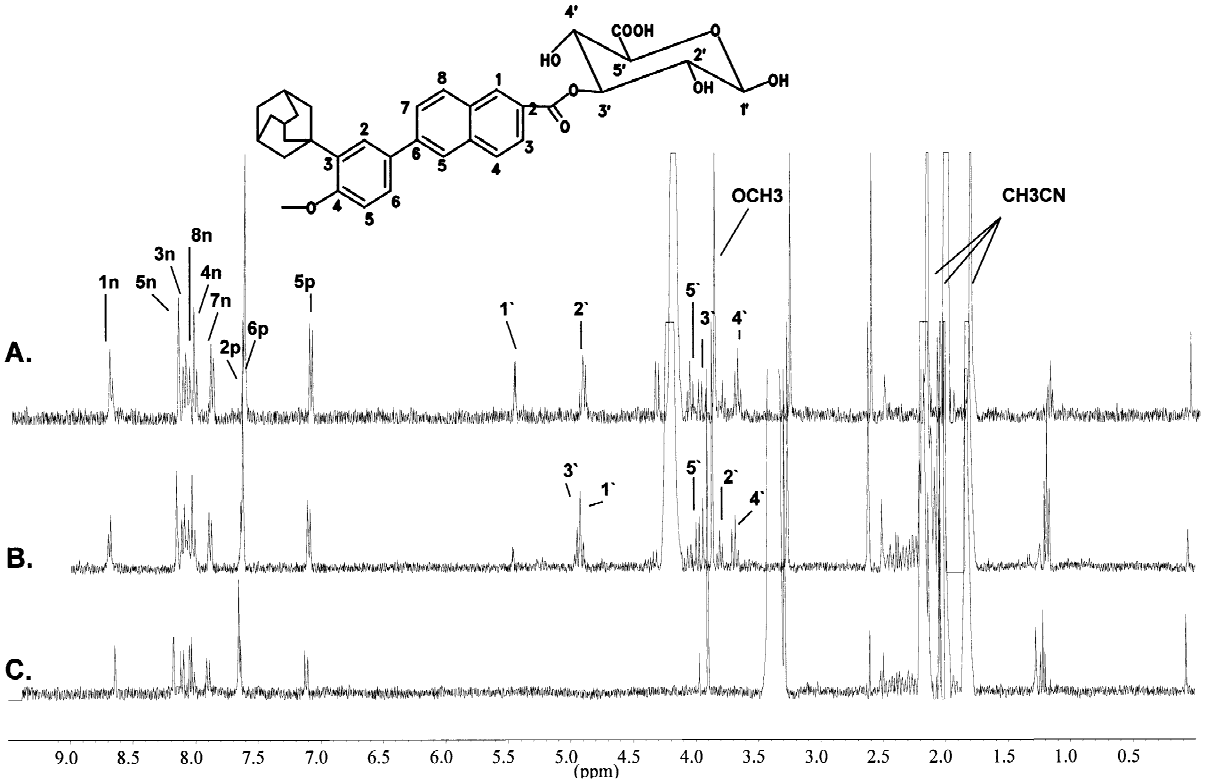

Fig. 3. ^{1}H-NMR spectra of (A) 2′-CD271G, (B) 3′-CD271G and (C) CD271. In spectrum A the protons of CD271 are marked, in the spectra A and B the protons of the glucuronic acid part of the CD271-glucuronide are marked. The positions of the protons are additionally marked in the structural formula of 3′-CD271G. n – are indicated as protons of the naphthoic acid system; p, protons of the phenyl system. The protons of the sugar are marked additionally with an X′. In the spectra of A and B some disturbing peaks are seen which are due to contaminations caused by rearrangements during long time measurement.

pellet diet (Altromin 1324; Altromin, Lage, Germany) and tap water ad libitum. The animals were mated during a 2-h period in the morning. The following 24 h were regarded as gestational day 0 (GD 0). The general principles of laboratory animal care were followed as well as the current version of German law on the protection of animals.

2.4.1. Pharmacokinetics of CD271-glucuronide

NMRI mice of gestational day (GD) 11 were treated with 30 mg CD271GB/kg body mass. The DMSO solution (1 ml/kg) was applied subcutaneously (neck region). Four mice were treated for each time point and sacrificed after 0.5, 2, 6, 8, 12, 24 and 48 h.

2.4.2. Laboratory precautions

As retinol is sensitive to light, all experiments including animal treatment and analytical work were carried out under dim yellow light.

2.4.3. Sample collection

Treated animals were killed by decapitation after the selected time intervals. Plasma, kidney, skin (trunk region) and liver (as well as embryos for later pharmacokinetic studies) were collected and stored at −20°C. Maternal plasma was collected into heparinized funnels and heparinized 1-ml tubes. Plasma was prepared by centrifugation (10 min at 1500 *g* at 4°C). Tissues were quickly removed, the skin samples were shaved and cut out with scissors.

2.5. Analytical procedures

2.5.1. Sample pretreatment

About 80 μl of plasma were accurately diluted with a threefold excess of acetonitrile. After 3 min of shaking the precipitated protein was pelleted by centrifugation. Kidneys from one animal were pooled and diluted with one volume equivalent of water and minced with scissors. An 80-μl volume of this preparation was diluted with a threefold excess of isopropanol and disrupted by ultrasonic treatment on ice. Liver was diluted with a ninefold excess of ice-cold 0.9% aqueous NaCl solution and homogenized with a PTFE-glass potter. An 80-μl volume of the homogenate was diluted with a threefold excess of isopropanol and disrupted by ultrasonic treatment on ice. The skin was shaved with an electric razor and a piece of 2 cm×2 cm was cut out of the trunk region of the animals back. The skin was diluted with a threefold excess of buffer A (50 m*M* Tris–HCl, 25 m*M* NaCl, 25 m*M* EDTA, 1 m*M* dithiothreitol adjusted to pH 7.5), minced with scissors and disrupted by ultrasonic treatment on ice and 80 μl was diluted with a threefold volume of isopropanol [26]. After the treatment described above, the sample was centrifuged for 5 min and the supernatant (260 μl) was accurately diluted with one volume of 0.5% aqueous ammonium acetate solution and placed into the AS-4000 autosampler [26].

2.5.2. Determination of retinol, CD271 and CD271GB

Retinol, CD271 and CD271GB concentrations were determined using fully automated online solid-phase extraction and automated reversed-phase HPLC analysis [26].

3. Results

3.1. Structure determination of purified CD271G by HPLC–NMR coupling

Preparative reversed-phase HPLC resulted in a mixture of several glucuronide isomers (Fig. 2) which could be identified by HPLC–NMR coupling. Fig. 3 shows that purified CD271G consists essentially of CD271, 2′-CD271G and 3′-CD271G and possibly small amounts of 4′-CD271G and 1′-CD271G. The glucuronide positional isomers could be easily identified by the position of the anomeric proton 1′ of the glucuronide and the positions of the sugar proton, where the glucuronic acid is bound to the aglycone. The proton of the 2′-position of the 2′-CD271G occurred at 4.9 ppm in comparison to the proton of the 3′-position at 4.0 ppm (Fig. 3A). The proton of the 3′-position of the 3′-CD271G could be found at 4.9 ppm in comparison to the 2′-position proton at 3.8 ppm (Fig. 3B). The anomeric protons are shifted in the positional isomers from 5.5 ppm at the 2′-CD27G to 4.9 ppm at the 3′-CD271G.

Incubation of separated 3′-CD271G in the flow probe for different periods of time demonstrates its rearrangement to mainly 2′-CD271G (Fig. 4). A subfraction obtained after 40 min elution from the preparative column consisted mainly of 2′-CD271G and 3′-CD271G. This subfraction coded CD271GB was used for the pharmacokinetic experiments.

3.2. Pharmacokinetic study of CD271GB

Maximum concentrations and area under the curve (AUC) values for CD271GB were found in the liver (C_{max} 12 676±2595 ng/g) which were ~20 times higher than in plasma (C_{max} 684±270 ng/ml) (Table 1 and Fig. 5). The observed variability of the data is due to the way of treatment (subcutaneously) and the small group size (n=4 per time-point). Enlargement of the group size would have reduced the standard deviation but would not change the observed concentration–time curve. Therefore, we reduced the amount of animals to the minimum in accordance with the German Animal Protection Law.

3.3. Effects of the retinoids on the endogenous retinol level in plasma

CD271GB treatment led to a reduction of the retinol plasma level from 39.5±9.0 ng/ml at 0.5 h regarded as a baseline level to 16.9±2.6 ng/ml at 8 h. 48 h after application of the compound the original plasma level was restored (Table 2).

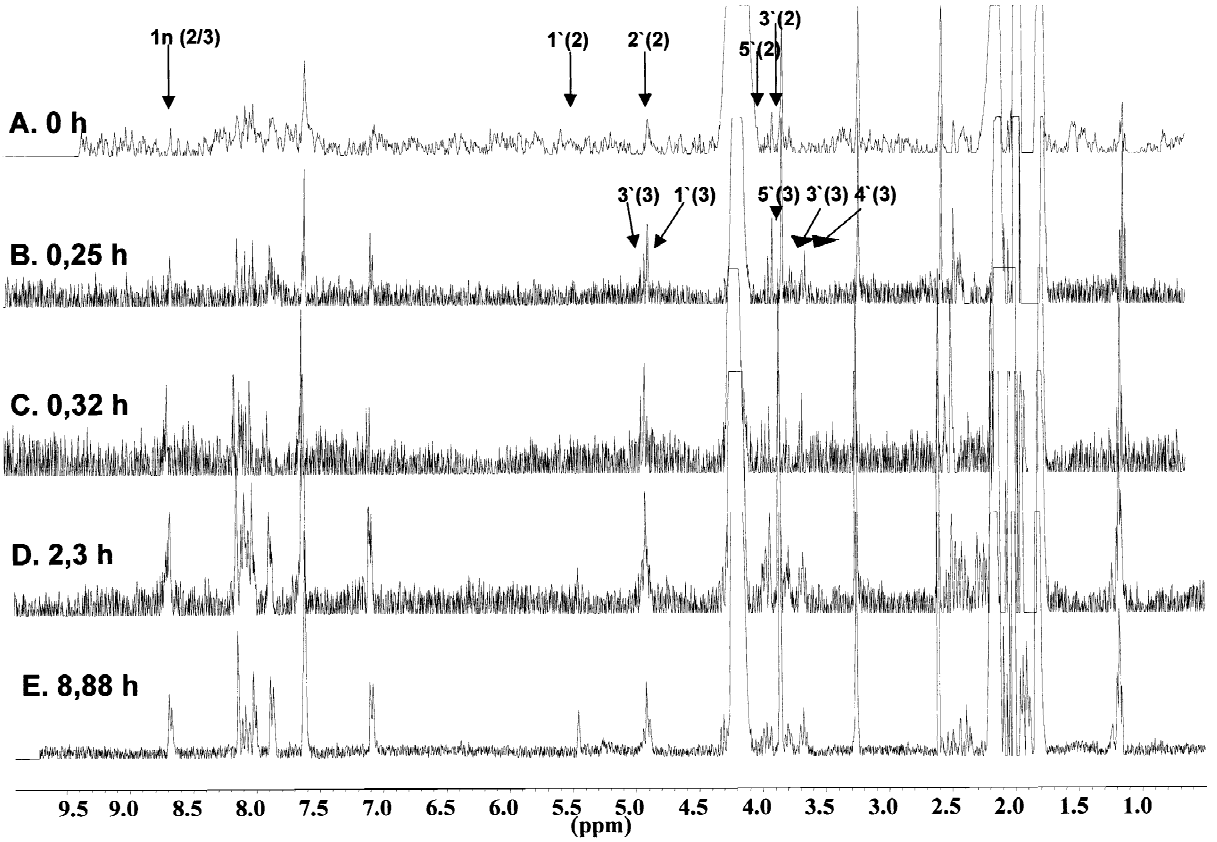

Fig. 4. ^{1}H-NMR of CD271G after direct ''stop flow'' measurement in the NMR flow probe after HPLC separation. In spectrum A the expected proton shift positions of 2′-CD271G are shown and in spectrum B the proton shifts of the source substance 3′-CD271G. After (A) 0 h, (B) 0.25 h, (C) 0.32 h, (D) 2.3 h, (E) 8.88 h the substance was measured again. Rearrangements of 3′-CD271G in the NMR flow cell could be determined.

Table 1
$AUC_{(0.5-48\ h)}$ (mean±SD) and C_{max} (mean±SD) of CD271GB and CD271 after subcutaneous injection of 30 mg CD271GB/kg in pregnant mice (n=4) (gestation day 11)

	C_{max} (ng/ml or ng/g)	t_{max} (h)	AUC (ng h/ml or ng h/g)	
Plasma	684±270	6	10 857	CD271GB
Skin	900±339	8	12 690	
Kidney	1279±91	8	19 949	
Liver	12 676±2595	8	162 002	
Plasma	69.9±7.0	8	1355	CD271 (as a metabolite)
Skin	108±50	8	3700	
Kidney	177±9	6	3152	
Liver	423±121	6	7469	

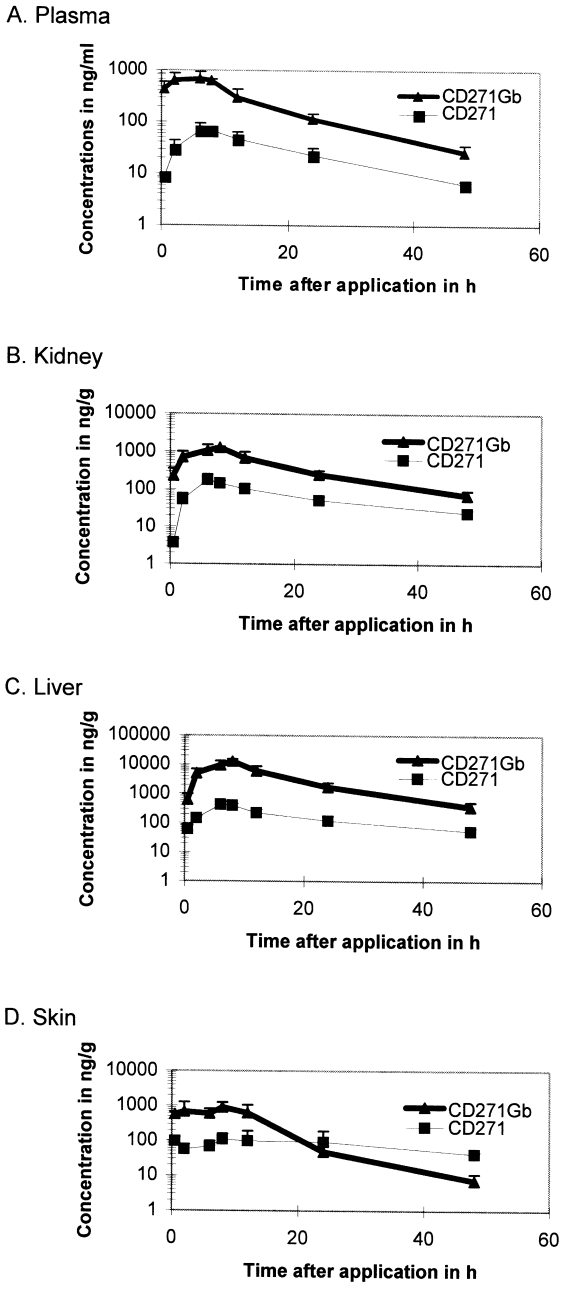

Fig. 5. Concentrations of CD271GB and CD271 in (A) plasma, (B) kidney, (C) liver and (D) skin after subcutaneous injection of 30 mg CD271GB/kg body mass in pregnant mice of gestational day 11 (n=4).

4. Discussion

Glucuronides are common metabolites of several natural and synthetic retinoids. Since glucuronidation of a compound may improve its pharmacokinetic properties and ensure its continuous release over a longer period of time our goal was to synthesize and characterize the glucuronide of CD271, a drug used for the topical treatment of mild to moderate Acne vulgaris.

Since glucuronides tend to rearrange via acyl migration to result in a mixture of positional isomers [13,14], coupling of NMR to an HPLC system was necessary for identification and separation purposes. The developed HPLC method had to fulfill two purposes: (i) to separate each of the acyl isomers in the equilibrium mixture (Fig. 4) and (ii) to provide a solvent composition that is NMR suitable since their ^{1}H-NMR signals determine by their chemical shift the regions of the spectrum that are available for the detection of signals of the sample molecule. Therefore the use of solvents with only a few sharp signals was advisable. For this reason we chose a gradient of acetonitrile/$^{2}D_{2}O$. The separation of a mixture of positional isomers and the metabolite CD271 is shown in Fig. 2.

Identification of two isomer peaks was achieved by HPLC–NMR coupling and all ^{1}H-NMR spectra were recorded in the stopped-flow mode. In Fig. 3 the ^{1}H-NMR spectra of 2′- and 3′-CD271G are summarized with assignment of the signals to the protons. The main components of the purified CD271-glucuronide (3′-CD271G and 2′-CD271G) eluted very close to each other. However, no baseline separation was necessary for their identification and structural elucidation. The acyl migration of these CD271-glucuronides forming different position isomers is shown in Fig. 6. To investigate the stability of 3′-CD271G towards rearrangement in the solvent, the compound was stopped immediately after HPLC separation in the flow cell of the NMR and measured at several incubation times. Fig. 4 shows the ^{1}H-NMR spectra thus recorded. By comparing the spectra, the rearrangement of 3′-CD271G to 2′-CD271G becomes visible after 2.3 h. Due to rearrangement after purification a position-isomer mixture (named CD271GB) has been used for the pharmacokinetic experiments. It has been concluded

Table 2
Retinol concentration in mouse plasma (n=4) after subcutaneous injection of 30 mg CD271GB/kg on day 11 of gestation (mean±SD)

Time after	After CD271GB		
administration (h)	Retinol (ng/ml)	CD271GB (ng/ml)	CD271 (ng/ml)
0.5	39.5±9.0	438±167	8.89±0.76
2	36.6±5.5	653±219	30.0±14
6	21.9±2.8	684±270	68.3±24.9
8	16.9±2.6	646±44	69.9±7.9
12	25.8±2.7	299±148	46.5±17.4
24	25.9±6.4	113±33	22.3±9.1
48	39.8±0.8	26.4±8.7	64.8±0.85

that the used solvent is appropriate for separation, but not for preventing positional rearrangement after long incubation time of the substance in the solvent. Usage of the positional isomers (3′-CD271G to 2′-CD271G) for in vivo experiments has been show to be acceptable undergoing a steady release in the organism to yield high amounts of CD271 as the active drug.

In a previous study ATRAG was administered orally and the level of retinoids was determined in maternal plasma. No changes in the concentration of endogenous retinoids as well of ATRAG could be detected. The reason for this could be that the polar and water-soluble ATRAG could not be absorbed due to its physicochemical properties and/or the oily vehicle reduced its absorption [27]. In another set of experiments ATRAG was administered intravenously or subcutaneously leading to high levels of ATRAG and the hydrolysis product all-*trans*-retinoic acid in the plasma [10].

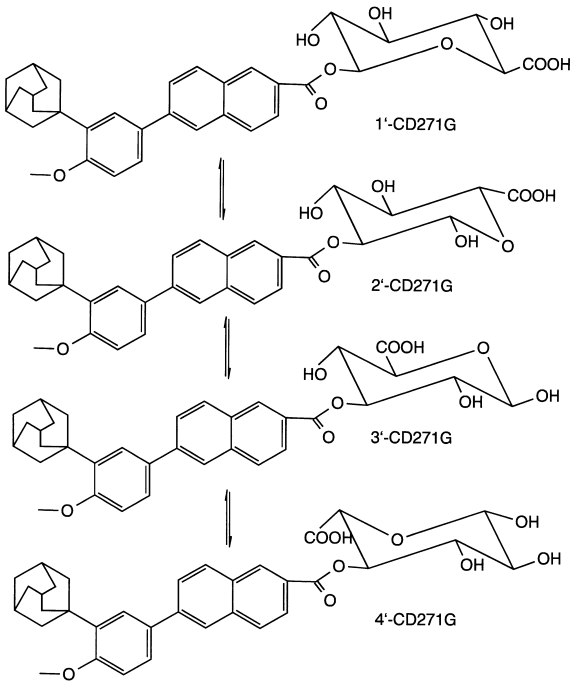

Fig. 6. Structural formulas of several position isomers of CD271-glucuronide.

In the present study CD271GB was therefore applied subcutaneously to NMRI-mice at a single dose of 30 mg/kg in DMSO. The concentrations of CD271, CD271GB and retinol were determined in plasma, skin, kidney and liver of the sacrificed mice at various time points (0.5, 2, 6, 8, 12, 24 and 48 h). CD271GB appeared in all analyzed compartments. The highest AUC values for both CD271G and CD271 were found in the liver.

The biological effects of retinoids may not only be caused by their interaction with the nuclear retinoid receptors, but also by their interference with the function and/or metabolism of endogenous retinol [28–31]. This observation prompted us to study the effect of CD271 and CD271GB on retinol plasma levels. Both compounds decrease the plasma-concentration of retinol. This decrease points into the direction of a feedback mechanism: the endogenous plasma concentration of retinol, the precursor of the active RAR binding molecule all-*trans*-retinoic acid, seems to be under the control of retinoic acid receptor agonists [32] or under control of the retinol binding protein [33].

Our results show that CD271GB could be well detected in plasma or several organs following

subcutaneous administration. Significant concentrations of its parent compound CD271 can be recovered from plasma and tissue indicating that this novel glucuronide – like ATRAG – can be hydrolyzed considerably in vivo if absorbed into the systemic route. Further experiments have to show whether topical administration of CD271GB offers an advantage, such as reduced skin irritation, over CD271 in the treatment of acne.

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