Fully automated determination of selective retinoic acid receptor ligands in mouse plasma and tissue by reversed-phase liquid chromatography coupled on-line with solid-phase extraction

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Abstract

A fully automated reversed-phase HPLC method was developed for the quantitative assay of three retinoids (Am-580, CD-2019 and CD-437) which selectively activate the retinoic acid receptors RARα, RARβ and RARγ, respectively. Mouse plasma, embryo and maternal tissues were prepared for injection by on-line solid-phase extraction (SPE) and valve-switching techniques. Following automatic injection, the sample was loaded on preconditioned disposable cartridges, cleaned-up and then transferred onto the analytical column to be eluted in the backflush mode, separated by gradient elution and detected by UV, while a new cartridge was concomitantly conditioned. The overall recovery was quantitative allowing for external standardization. The calibration curves were linear in all biological samples tested so far, with a correlation coefficient (r) >0.99. The intra-day precision was ≤7.8% ($n=5–6$) and the inter-day variability was ≤9.4% ($n=3$). The lower limit of detection was 2.5 ng/ml or ng/g for CD-2019 and CD-437, and 5 ng/ml for Am-580 with a S/N ratio of 5 using a sample weight of 25 μl or mg. The method is now in routine use in our laboratory for the assessment of the pharmacokinetic profiles of these retinoids. The small sample size required, the simple sample prepration and the rapid analysis with high degree of automation make this method convenient for microanalysis of biological samples both in animal and human studies.

Keywords: Sample preparation; Retinoids; Retinol

1. Introduction

Retinoids, natural and synthetic analogs of vitamin A alcohol (retinol; ROH), are implicated in a wide variety of key biological processes including cell growth and differentiation, embryogenesis and epithelial homeostasis [1,2]. Clinically, retinoids have proven efficacy in the treatment of dermatological diseases such as acne, psoriasis and photoaged skin

[3,4], as well as in certain malignancies [5]. The molecular mechanisms of such compounds are thought to be mediated, for the most part, through regulation of gene expression by two subclasses of nuclear retinoic acid receptors RARs and RXRs [6,7]. Both of these subclasses contain three receptor subtypes arbitarily designated α, β, and γ, which are encoded by separate genes [8].

The clinical usefulness of retinoids is limited by a number of side effects such as bone and lipid toxicities [9], and teratogenicity [10,11]. Therefore, new retinoid congeners with superior therapeutic

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indices compared to the currently used retinoids, would be highly desirable [12]. Since the retinoid receptors have distinct spatial and temporal distribution patterns [13,14], and because the target gene specificities of the receptors are different, it has become clear that independent response pathways could be elicited by retinoid analogs of different receptor selectivity. Therefore, it would be expected that receptor-selective ligands would provoke more restricted responses than their non-specific counterparts [13]. In this respect, three receptor-selective ligands were synthesized namely, Am-580 (α-ligand), CD-2019 (β-ligand) and CD-437 (γ-ligand), and have shown biological activity comparable to, or higher than retinoic acid in murine F9 teratocarcinoma cell line [12,15]. However, they proved to be teratogenic in mice with a potency order $\alpha > \beta > \gamma$ [31]. Here, the malformations induced by the α-ligand were qualitatively similar to, but more severe than those produced by all-*trans* retinoic acid (RA) (37.5 mg/kg body weight). However, the teratogenic potency of RA was more than that of either CD-2019 or CD-437. Pharmacokinetic studies are therefore important for drug monitoring [16] as well as the interpretation and design of teratogenicity experiments of retinoids in an attempt to extrapolate the experimental findings to the human (risk assessment), as well as to elucidate whether the parent retinoid drug or a metabolite is the proximate or ultimate teratogen [17,18].

In the current study, we have developed a fully automated reversed-phase HPLC method for the determination of all the three selective ligands in addition to endogenous ROH levels in mouse plasma, embryo and maternal tissues using on-line solid-phase extraction (SPE) on exchangeable cartridges. Actually, liquid chromatography (LC) with ultraviolet (UV) detection is the method of choice for the determination of retinoids in biological samples, since it is rapid, sensitive and allows the separation of many isomers and metabolites within a wide polarity range [19]. Since the first commercial SPE columns were introduced in 1978 [20], the use of SPE techniques for the isolation of drugs from various biological samples has rapidly increased [21]. In recent years, the development of automatic SPE instruments has taken the SPE techniques to a higher level of experimental precision and laboratory safety as well as higher throughput [22].

Our method differs from the previous SPE methods in that, sample preparation is achieved on-line compared to other off-line methods [23,24]. In such other methods, the samples are prepared for injection into the HPLC system by the Varian AASP module which is an automated device for SPE using cartridges (packed with bonded silica) followed by on-line HPLC analysis. In our method however, only the first pretreatment step (tissue homogenization and sample extraction with acetonitrile) is carried out off-line. In addition, the sample enrichment, clean-up and transfer onto the analytical column is carried out using valve-switching techniques rather than column-switching techniques as described in other on-line methods [19,25]. We have used this method effectively for the evaluation of the transplacental pharmacokinetics of the retinoid ligands as well as their disposition in various maternal tissues.

2. Experimental

2.1. Chemicals

CD-2019 [6-{3-(1-methylcyclohexyl)-4-methoxyphenyl}-2-naphthoic acid], CD-437 [6-{3-(1-adamantyl)-4-hydroxyphenyl}-2-naphthoic acid] [26] and Am-580 [{4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl) carboxamido} benzoic acid] [27] (Fig. 1), were kindly provided by Dr. B. Shroot and Dr. U. Reichert (CIRD-Galderma, Sophia Antipolis, Valbonne, France). Vitamin A alcohol (retinol) was obtained from Serva (Heidelberg, Germany). Cremophor EL® and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade) as well as acetic acid, isopropanol, ethanol and ammonium acetate (analytical grade) were all purchased from Merck (Darmstadt, Germany). Water was deionised and purified by a Milli-Q system (Millipore, Eschborn, Germany).

2.2. Laboratory precautions

Although the synthetic retinoids are stable and light-insensitive, all manipulations were carried out in darkened room with dim yellow light to avoid the degradation of ROH. The autosampler was also
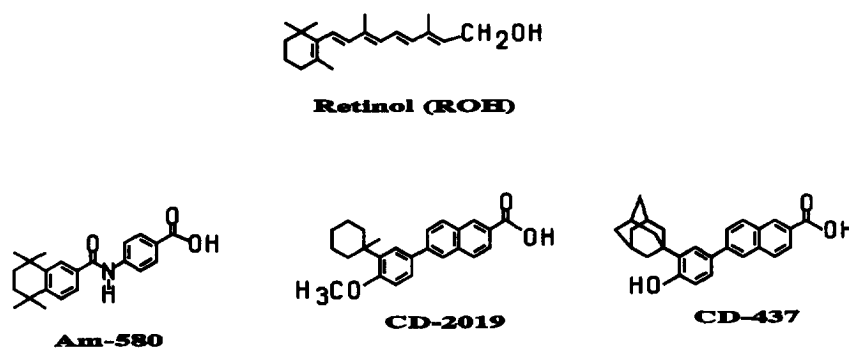
Fig. 1. Chemical structures of retinol (ROH) and the three selective ligands included in this study

equipped with a thermostatically controlled sample tray (4°C).

2.3. Animal experiment

NMRI Mice (Han, Zentralinstitut für Versuchstierkunde, Hannover, Germany) were given each retinoid separately on day 11 of gestation (plug day= 0) as a single intragastric dose of 15 mg/kg body weight, in aqueous suspension of Cremophor EL (25% w/w), in a dosing volume of 5 ml/kg. The animals were then sacrificed at various time intervals. Plasma, embryos and maternal tissues were collected and stored at −20°C prior to analysis. Plasma was obtained from retro-orbital blood samples [withdrawn under light ether anaesthesia using heparinized microcapillaries (Brand, Wertheim, Germany)] by centrifugation at 4°C for 5 min at 3000 g (Heraeus Christ Minifuge 2, Berlin, Germany).

2.4. Chromatographic system

The HPLC system (Fig. 2) consisted of two HPLC pumps type LC-10AD (Solvent delivery module with a double plunger reciprocating pump) connected to a system controller type SCL-10A and a dynamic mixing chamber (Mixer SUS; P/N 228-28000-91), a two-channel type SPD-10AV UV detector and a C-R4AX Chromatopac integrator (all from Schimadzu, Duisburg, Germany). The eluents were degassed using a DEGASYS DG-1310 (VDS Optilab, Berlin, Germany) prior to mixing and then passed through an on-line filter (1–2 μm) (Knauer, Berlin, Germany) before reaching the analytical column (120×4 mm I.D.), which was packed in our laboratory with

Spherisorb OD2 3 μm (Phase Separation, Deeside, UK) and embedded in a blockheater (VDS Optilab, Berlin, Germany). A multilinear binary gradient was formed from solvent A [60 mM aqueous ammonium acetate–methanol (1:1,v/v)] and solvent B (pure methanol) as previously described by Eckhoff and Nau [28] (Table 1). However, we used solvent A without adjusting the pH to 5.75 as in the previous study. The flow-rate was adjusted to 0.7 ml/min and the column was heated to 60°C. This method (HPLC-system I) was used for the analysis of CD-2019 and ROH at 318 nm. It was modified (by adjusting the pH of solvent A to pH 4.5 with acetic acid) to allow the separation of both CD-437 and Am-580, which were detected at 323 and 285 nm, respectively (HPLC-system II). When Am-580 was measured at 285 nm, ROH was concurrently detected at 325 nm using the dual wavelength detection mode. The HPLC unit was optionally equipped with both Model 7125 manual injector and Model 7000 switching valve (Rheodyne, Cotati, CA, USA), which allowed manual as well as automatic sample injection.

2.5. Solid-phase extraction

Sample enrichment and clean-up were achieved using an on-line SPE module, consisting of a Model L-6200A low pressure intelligent pump with a proportionating valve working for the time-sharing delivery of mobile phase components, and an intelligent autosampler Model AS-4000 equipped with a cooling system and operating in a sequential mode (all from Merck, Darmstadt, Germany). The autosampler was connected to a programmable on-line
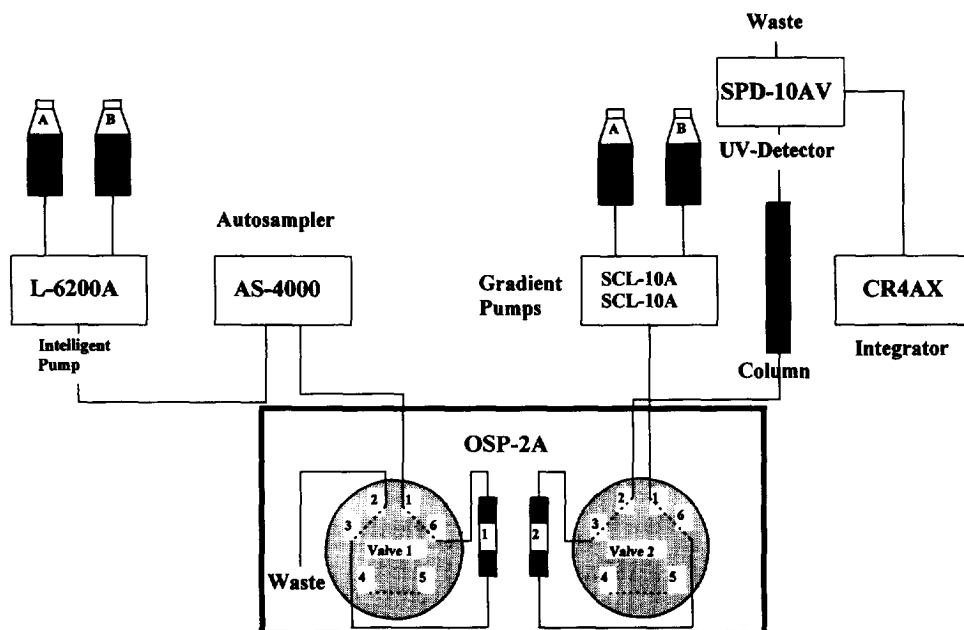
Fig. 2. Schematic diagram for the HPLC unit coupled on-line with the SPE module working in the backflush mode.

SPE unit (Sample preparator; OSP-2A; Merck, Darmstadt, Germany) that provided a fully automated sample clean-up step which was on-line incorporated into the HPLC analysis system. The instrument contained a cartridge magazine in the form of a ring loaded with up to 72 small prepacked extraction columns (OSP-2A cartridges, LiChrospher 60; RP-18, 10 μm). The ring movement was controlled by two separate independently moving switching valves (valve 1 and valve 2) which were operated pneumatically.

Table 1
Composition of mobile phase (multilinear binary gradient)

Time (min)	A[a](%)	B[b](%)
0.0	85	15
11	10	90
11.2	1	99
12	1	99
12.5	85	15
15	85	15

[a]Solvent A: 60 mM aqueous ammonium acetate–methanol (1:1, v/v).
[b]Solvent B: Pure methanol.

2.6. Sample preparation

All steps of sample preparation were performed on-line except for the first pretreatment step where 30–100 μl or mg of the biological samples (plasma, embryo and maternal tissues) was extracted with a three-fold volume of acetonitrile, vortexed for 1 min, shaken for 3 min, and centrifuged at 4000 g for 4 min at 4°C using Heraeus Biofuge 15R (VDS Optilab, Berlin, Germany). The tissues were disrupted before centrifugation by sonication on ice using a B-12 sonifier at setting 2.5 (Branson Sonic Power Company, Danbury, CT, USA). The supernatant (100 μl) was then automatically injected into the HPLC system. Prior to sample injection, the cartridges (15×4 mm) were conditioned with 9 ml of acetonitrile and equilibrated with 2.5 ml of aqueous buffer (ammonium acetate; NH_4OAc, pH 4) to receive the sample. When the autosampler injected the programmed sample volume, the time program of the gradient pump was simultaneously started. The time events (eight time-controlled potential-free switching contacts), were defined in the time program of the L-6200A pump and controlled and

synchronized all OSP-2A functions. These functions (valve 1, valve 2, clamp and direction) were controlled or activated by those contact closures in the 'REMOTE' operating mode and the function 'MOVE' was activated by a pulse signal sent by the pump. The actual chromatographic analysis was started by the transfer of the cleaned-up sample from the preparation side to the elution side of the OSP-2A where, it was eluted in the backflush mode. In the meantime, the intelligent pump started the data collection at the integrator, while on the preparation side a new cartridge was being conditioned. Table 2 shows the time program that had been created in the L-6200A pump. After all the cartridges had been processed, the ring had to be manually reloaded.

2.7. Standard solutions

Stock solutions of all the three retinoids were prepared by dissolving 2.5 mg of each in 25 ml of ethanol to give a final concentration of 100 μg/ml. ROH was dissolved in isopropanol (1 mg/ml). All stock solutions were stored at $-20°C$ and serial dilutions were freshly prepared using ethanol.

2.8. Method validation

The reference retinoids used in the assay validation were all prepared by spiking BSA solutions (5% w/v) with known amounts of the alcoholic standards (Am-580, CD-2019, CD-437, and retinol). For recovery purpose, three different concentrations (50, 500 and 5000 ng/ml) were chosen to cover the expected retinoid levels in plasma and tissues. Recovery was determined by comparing the peak areas obtained from spiked samples ($n=6$) with those from manual injections ($n=6$) of matrix-free alcoholic solutions containing respective retinoid concentrations. Reproducibility (precision) was estimated as % coefficient of variation (C.V. %) of replicate sample analysis over three consecutive days. Both the intra-day (within-day) ($n=5–6$) and inter-day (between-day) ($n=3$) assay precision data were calculated and assay results with $\leq10%$ C.V. were accepted. For determination of the lower limit of detection, a signal to noise ratio (S/N) of 5 was considered. Linearity of a wide range of concentrations (25–5000 ng/ml) was tested. The calibration curves were obtained by linear regression analysis. The concentration range in which the method was linear, was chosen for the

Table 2
Program for automatic (on-line) sample preparation and injection by use of the valve-switching technique

Time (min)	Solvent A[a](%)	Solvent B[b](%)	Flow-rate (ml/min)	Events	Remarks
0.0	85	15	0.0		Sample injection by autosampler
0.1	85	15	1.0		
2.1	85	15	1.0		
2.2	0.0	100	0.0	10 20	Both valves (1 and 2) are switched to position 1 (home position)
2.3	0.0	100	0.0	30	The clamp is opened
2.4	0.0	100	0.0	42	The ring moves one step
2.5	0.0	100	1.0	31	The clamp is closed
2.6	0.0	100	1.0	21	Valve 2 is switched to position 2
2.7	0.0	100	1.0	72 82	Starting signal for the HPLC, both the pumps and the integrator, respectively
2.8	0.0	100	3.0	11	Switching of valve 1 to position 2 Start of preconditioning
5.8	0.0	100	3.0		
5.9	100	0.0	0.0	10	Home position of valve 1 (end of preconditioning)
6.0	100	0.0	0.5	11	Start of equilibration step
11.0	100	0.0	0.5	10	End of equilibration
16.5	0.0	0.0	0.0	11	The cartridge is ready for new sample injection

[a] Solvent A; aqueous ammonium acetate buffer (60 mM, pH 4).
[b] Solvent B; pure acetonitrile.

quantitative analysis. For quantitative assay of the retinoids in different biological samples, a calibration run was made using BSA solution spiked with retinoids at three concentration levels (50, 500 and 5000 ng/ml). The calculation of response factors and coefficient of correlation was performed automatically by the integrator. Calibration curves were also determined in plasma and tissues. In general, calibration graphs were only accepted when the correlation coefficient was >0.99.

3. Results

Fig. 3a, Fig. 4a and Fig. 5a show standard chromatograms of BSA solutions spiked with the reference retinoids. All the three selective ligands and retinol were well detected. Carry-over was avoided because the cartridges were used once only. However, if the cartridges were to be used several times, purging with 9 ml of acetonitrile and then with 2.5 ml of aqueous buffer (60 mM NH$_4$OAc, pH 4), which was an integral step during sample preparation, was found efficient to prevent the memory effects.

3.1. Recovery

The assay recovery was assessed using BSA solution, mouse and human plasma as well as embryonal and maternal tissue homogenates spiked with known concentrations of the standard retinoids (50, 500 and 5000 ng/ml or ng/g). The percentage recovery of retinoids, Table 3, was calculated by analyzing replicate ($n=6$) spiked samples and comparing the peak areas with those obtained by direct injection of ethanolic solutions ($n=6$) containing respective amounts of the retinoids. The overall recovery was >90% for most of the samples analyzed by both HPLC-systems I and II.

3.2. Precision (reproducibility)

The assay precision over three consecutive days (for both HPLC-systems I and II) is shown in Table 4. The overall intra-day reproducibility was ≤7.8%, while the inter-day variability was ≤9.4%.

3.3. Limit of detection (assay sensitivity)

As little as 2.5 ng/ml or ng/g of all the retinoids except for Am-580 (5 ng/ml or ng/g) could be detected using a sample weight of 25 μl or mg.

3.4. Linearity and calibration

Our method (I and II) was linear for all retinoids in the range of 50-5000 ng/ml or ng/g in all the sample matrices tested so far with a correlation coefficient (r) >0.99. This, in addition to the good recovery, allowed for external standardization where BSA solution was spiked with all the retinoids at concentrations 50, 500 and 5000 ng/ml.

3.5. Experimental application

Figs. 3-5 show typical chromatograms of plasma and embryo homogenate from pregnant mice (gestational day 11) at 2 h following treatment with 15 mg/kg body weight of each retinoid. Due to the close similarity of the absorption maxima of both CD-2019 (318 nm) and CD-437 (323 nm) with that of ROH (325 nm), retinol could be detected in the same run with both retinoids at 318 and 323 nm, respectively. However, when Am-580 was analyzed (absorption maximum=285 nm), ROH was detected concurrently at 325 nm using the dual wavelength detection mode displayed on the two channels of the integrator.

4. Discussion

The HPLC methods (I and II) described above allow for the quantitative determination of the selective retinoid ligands included in this study, as well as the endogenous ROH levels in mouse plasma, embryonal and maternal tissues. Simplification of sample pretreatment requires sophisticated chromatographic instrumental set-up, which offers a new perspective for automation [29]. This holds true for our method, since the sample is simply mixed with a three-fold volume of acetonitrile and centrifuged (after tissue sonification) followed by direct injection of the supernatant into the HPLC system. Except for this simple pretreatment step, the method is fully
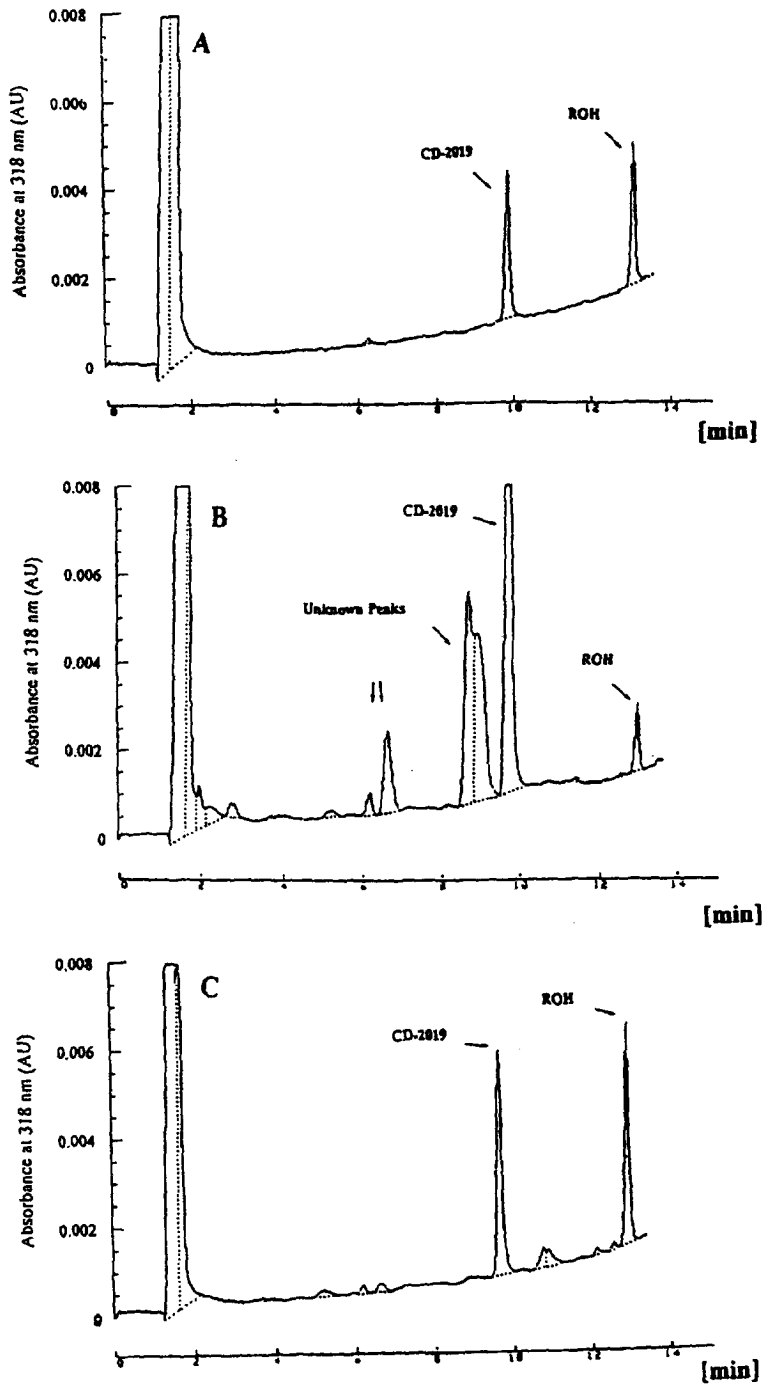
Fig. 3. Chromatograms of CD-2019 and ROH in (A) BSA (each 50 ng/ml); (B) mouse plasma; and (C) embryo homogenate. Samples were collected from pregnant mice (gestational day 11) 2 h following a single oral dose of CD-2019 (15 mg/kg body wt.). Sample weight was 25 μl or mg. Actual samples (30–100 μl or mg) were diluted with acetonitrile (1:3) sonified (for embryo), centrifuged and 100 μl of the extract was injected. Detection at 318 nm with 0.005 AUFS (HPLC-system I). Plasma retinoid concentrations: CD-2019, 1320 ng/ml; ROH, 27 ng/ml. Embryo retinoid concentrations: CD-2019, 172 ng/g; ROH, 77 ng/g.
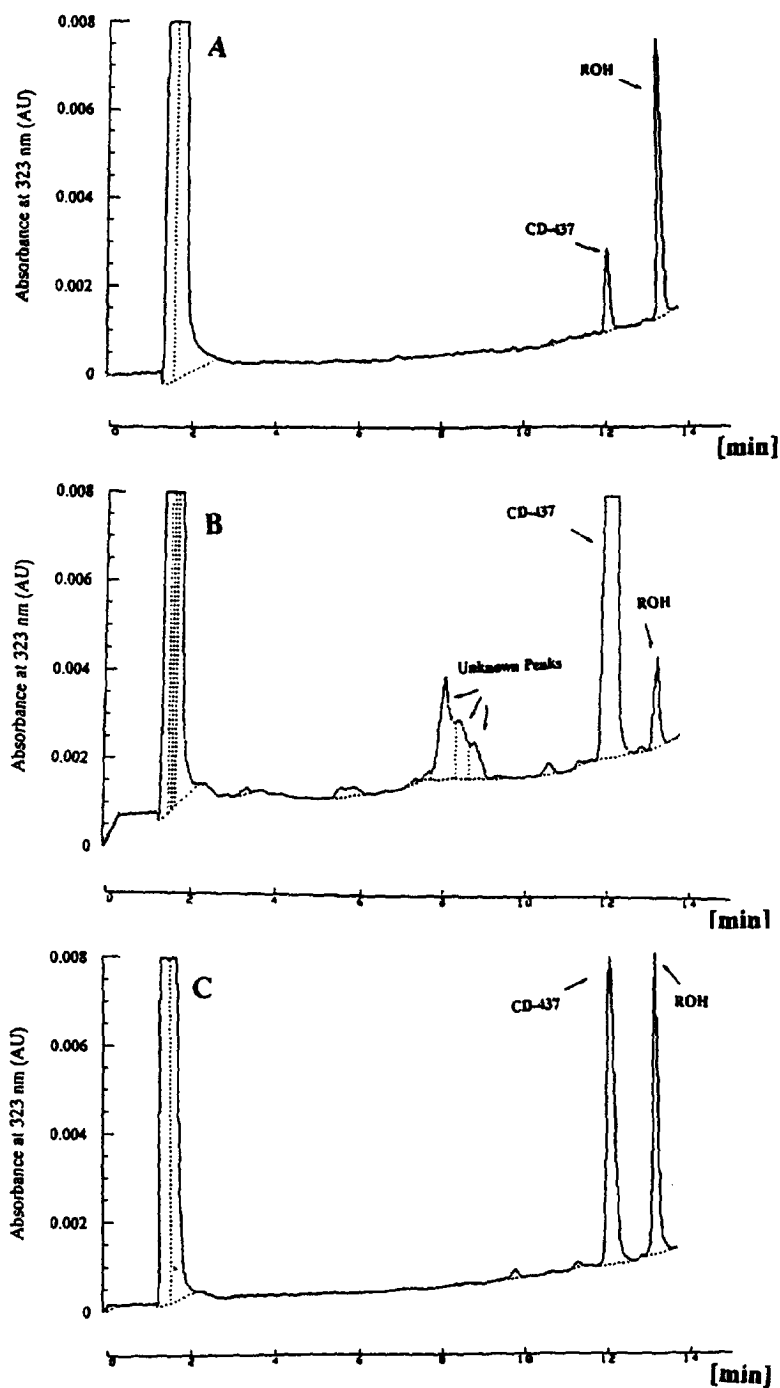
Fig. 4. Chromatograms of CD-437 and ROH in (A) BSA (each 50 ng/ml); (B) mouse plasma; and (C) embryo homogenate. Samples were collected from pregnant mice (gestational day 11) 2 h following a single oral dose of CD-437 (15 mg/kg body wt.). Sample weight was 25 μl or mg. Actual samples (30–100 μl or mg) were diluted with acetonitrile (1:3) sonified (for embryo), centrifuged and 100 μl of the extract was injected. Detection at 323 nm with 0.005 AUFS (HPLC-system II). Plasma retinoid concentrations: CD-437, 4339 ng/ml; ROH, 13 ng/ml. Embryo retinoid concentrations: CD-437, 830 ng/g; ROH, 147 ng/g.
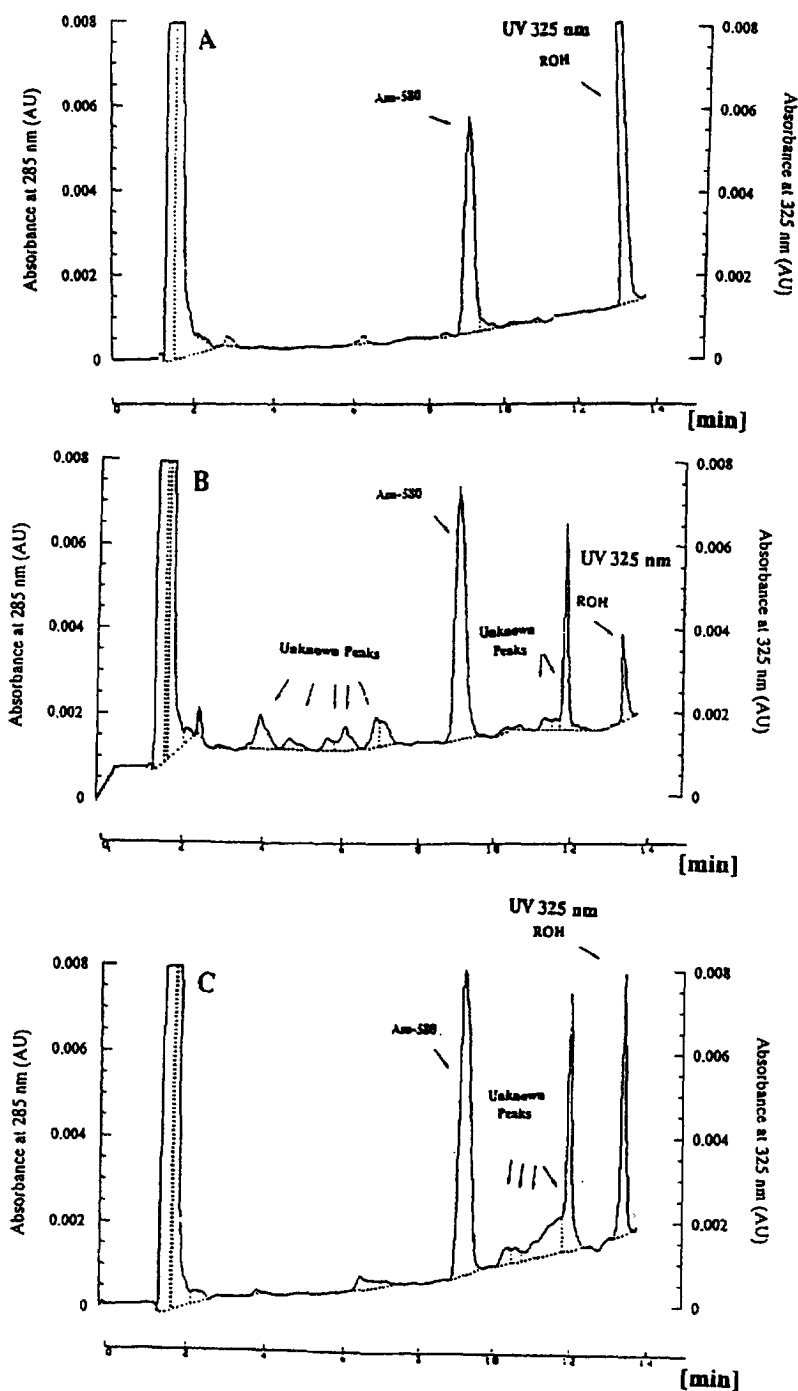
Fig. 5. Chromatograms of Am-580 and ROH in (A) BSA (each 500 ng/ml); (B) mouse plasma; and (C) embryo homogenate. Samples were collected from pregnant mice (gestational day 11) 2 h following a single oral dose of Am-580 (15 mg/kg body wt.). Sample weight was 25 μl or mg. Actual samples (30–100 μl or mg) were diluted with acetonitrile (1:3) sonified (for embryo), centrifuged and 100 μl extract was injected. Detection at 285 nm (Am-580) and 325 nm (ROH) with 0.005 AUFS (HPLC-system II). Plasma retinoid concentrations: Am-580, 735 ng/ml; ROH, 18 ng/ml. Embryo retinoid concentrations: Am-580, 929 ng/g; ROH, 149 ng/g.

Table 3
Assay recovery of CD-2019, CD-437, Am-580 and retinol

Retinoid	Concentration (ng/ml or ng/g)	Recovery (%)							
		BSA	Mouse plasma	Human plasma	Embryo	Placenta	Brain	Kidney	Liver
Retinol	50	95(97)[a]	105(95)	97(95)	90(89)	88(87)	88(87)	85(74)	93(89)
	500	97(98)	98(98)	99(98)	94(90)	86(93)	92(87)	91(81)	88(85)
	5000	95(93)	91(97)	94(92)	97(105)	90(96)	89(87)	90(86)	90(89)
CD-2019	50	98	93	99	94	99	104	88	93
	500	94	98	92	89	ND	93	92	93
	5000	97	98	98	ND[b]	93	92	90	90
CD-437	50	95	93	98	97	92	92	89	99
	500	95	96	93	97	99	99	96	94
	5000	92	93	92	113	102	96	97	98
Am-580	50	97	99	105	84	87	95	112	89
	500	99	104	106	95	110	102	102	107
	5000	95	94	100	91	90	93	98	93

[a] Data between brackets represent the assay recovery of retinol when analyzed by HPLC system II.
[b] ND=not determined.

Table 4
Assay reproducibility of CD-2019, CD-437 and Am-580 as well as retinol over three consecutive days expressed as coefficient of variation (C.V. %)

Retinoid	Concentration[a] (ng/ml)	Intra-day C.V. (%)			Inter-day C.V. (%) (n=3)
		First day (n=6)	Second day (n=5)	Third day (n=5)	
Retinol	50	4.6(3.1)[b]	6.5(1.7)	2.6(3.8)	6.2(2.3)
	500	3.3(1.7)	2.3(0.9)	3.5(4.6)	1.5(2)
	5000	3.7(1.7)	3.7(1.9)	3(3.3)	5.4(1.3)
CD-2019	50	2.1	3.4	2.2	5
	500	1.5	3.3	2	1.9
	5000	0.7	0.7	5.5	4.2
CD-437	50	7.8	2.6	2.3	9.4
	500	1.8	2.8	5.4	2.5
	5000	2.4	3.5	4.3	2.3
Am-580	50	5.4	1.5	3.3	3
	500	0.5	0.3	1.6	0.5
	5000	0.5	0.8	0.6	0.6

[a] Bovine serum albumin solutions were spiked with retinoid standards.
[b] Data between brackets represent the assay precision of retinol when analyzed by HPLC system II.

automated from the sample enrichment, clean-up, to direct transfer onto the analytical column.

The good recovery obtained (>90% for most of samples), allowed us to use external standards. Also the SPE step which was incorporated on-line with the autosampler in the HPLC system, shortened the lengthy extraction procedures which are normally used for chromatographing retinoids. This method increases the sample throughput, since a considerable number of samples could be automatically analyzed without operation intervention. Another benefit offered by the automation of the method is to ensure that all samples are processed the same way, thereby increasing the reproducibility of the assay results.

In contrast to CD-2019, both CD-437 and Am-580 initially suffered from peak irregularity (tailing and splitting) when analyzed by HPLC-system I. This was possibly related to the relative ionization during separation on the column. Since both ligands were more acidic and more polar than CD-2019, they might be ionized on the column, which had a nearly neutral pH, thus leading to secondary interactions on the sorbent. Therefore, we lowered the pH of solvent A. Optimal separation of both compounds was obtained with pH 4.5 (HPLC-system II). This might be ascribed, at least partly, to the reduced ionization in such an acidic milieu.

The assay sensitivity of the method was 2.5 ng/ml or ng/g for all the retinoids tested except for Am-580 (5 ng/ml), with sample weight of only 25 µl or mg. This was achieved in spite of the arotinoid structure of all the three selective ligands, which exhibit only half the UV extinction compared to the first and second generation retinoids [30]. A low limit of detection is of great importance because these arotinoids are highly active at very low concentrations (nM range). We are now testing if the sensitivity can even be further increased by the use of larger sample volumes.

The method was linear in the range of 50–5000 ng/ml or ng/g for all retinoids in all bio-samples (r>0.99). Calibration curves were made by spiking BSA solution with the retinoids at three concentration levels (50, 500 and 5000 ng/ml). The method has been used successfully in the pharmacokinetic study of these retinoids.

In conclusion, a fully automated HPLC method was developed using on-line SPE and valve-switching techniques. The high sensitivity achieved, the small sample volume, the simplicity of sample work-up with minimal handling and rapid analysis with high degree of automation make our method convenient and reliable for the microanalysis of retinoids in both experimental and clinical trials.

Acknowledgements

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