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Short Communication

Micro liquid chromatography–mass spectrometry with direct liquid introduction used for separation and quantitation of all-*trans*- and 13-*cis*-retinoic acids and their 4-oxo metabolites in human plasma

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ABSTRACT

The separation and quantitation of the pentafluorobenzyl derivatives of all-*trans*- and 13-*cis*-retinoic acids and their 4-oxo metabolites in human plasma on micro high-performance liquid chromatographic columns (0.32 mm I.D.) is described. The column outlet was directly coupled to the source of a quadrupole mass spectrometer via a simple SFC-frit interface. Negative ion chemical ionization conditions were obtained by coaxial introduction of ammonia as a reagent gas. A signal-to-noise ratio well above 3 was obtained for 1 pg of each analyte injected. The limit of quantitation calculated from spiked biological plasma extracts was 0.3 ng/ml.

INTRODUCTION

Isotretinoin (1) (Roaccutane®, 13-*cis*-retinoic acid) and tretinoin (2) (all-*trans*-retinoic acid) are important vitamin A analogues (Fig. 1), having a wide range of physiological functions. They are essential for vision, growth and reproduction, as well as for the differentiation of normal and neoplastic cells. Several studies have shown the antiproliferative activity of retinoic acids alone or in combination with other drugs, *e.g.* interferon-α [1]. In dermatology, retinoic acids are used in the treatment of cystic acne and photo damage [2]. Furthermore, retinoic acids can also stimulate changes in the immune systeme [3]. Therefore, retinoid status may play an important role in cancer prevention. On the other hand, it is well known that both isomers of retinoic acid and

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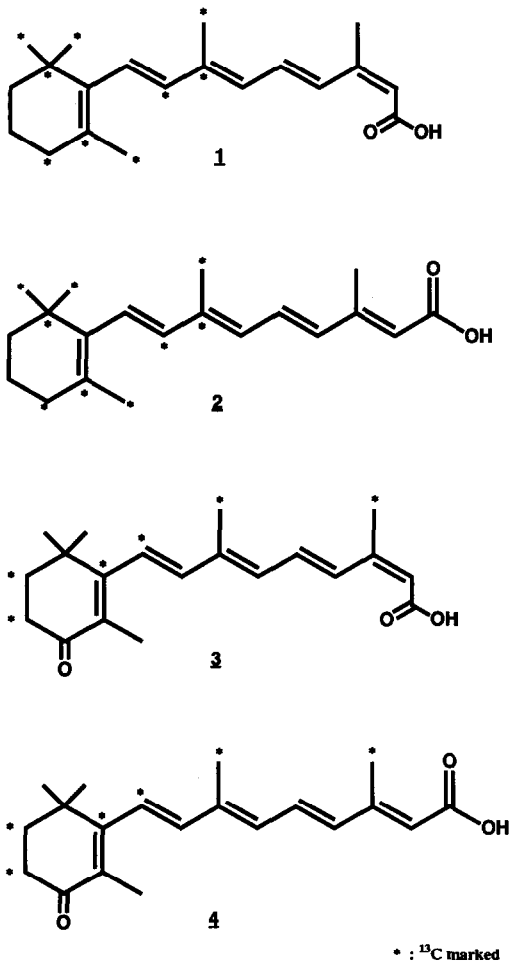

Fig. 1. Molecular structures of compounds **1–4**. ^{13}C-Labelled atoms are marked with asterisks.

their respective 4-oxo metabolites (**3** and **4**) are teratogenic [4]. It is therefore essential to determine the retinoid concentration in plasma both during and after therapy to indicate whether the retinoid status has been disturbed.

Several methods for the analysis of retinoids have been described, *e.g.* liquid–liquid extraction or solid phase extraction prior to high-performance liquid chromatographic (HPLC) analysis with UV detection, and gas chromatography–mass spectrometry (GC–MS) [5–7]. However, either the limit of quantitation of these methods lies above endogenous concentrations, or large volumes of plasma are needed. Only recently, an HPLC method with a limit of quantitation of 0.3 ng/ml for human plasma samples has been described, using on-line solid phase extraction with column switching [8].

Quantitation by HPLC–MS using stable isotope dilution techniques (^{13}C atoms as shown in Fig. 1) offers higher specificity of detection and, thus, greater accuracy than conventional HPLC–UV analysis. An HPLC–MS method has been published describing the quantitation of both isomers of retinoic acid and their 4-oxo metabolites in human plasma in the concentration range 0.5–32 ng/ml, using a Finnigan 3200 mass spectrometer, modified for better pumping, thus allowing for direct liquid introduction [9,10].

This paper describes a micro HPLC–MS method similar to the one described previously [9,10], using an unmodified Hewlett Packard HP 5988 mass spectrometer and a very simple and effective inlet system consisting of an SFC frit. A column of 320 μm I.D. is used to separate all the retinoids of interest. The limit of quantitation is 0.3 ng/ml for all analytes.

EXPERIMENTAL

Reagents

All reagents were either reagent-grade or HPLC-grade. Acetonitrile (ACN), ethanol, hexane, toluene and tetrahydrofuran (THF) were purchased from Merck (Darmstadt, Germany). Pentafluorobenzyl bromide (PFB) was purchased from Aldrich (Steinheim, Germany). Crown ether 18-crown-6 was purchased from Merck-Schuchardt (Hohenbrunn, Germany). Isotretinoin, tretinoin, 4-oxotretinoin and 4-oxoisotretinoin were obtained from Hoffmann-La Roche (Basel, Switzerland), and [9-^{13}C]isotretinoin, [9-^{13}C]tretinoin, 4-oxo[6-^{13}C]tretinoin and 4-oxo[6-^{13}C]isotretinoin were obtained from Hoffmann-La Roche (Nutley, USA).

Apparatus

The reversed-phase (RP) HPLC apparatus consisted of an AS 4000 Merck-Hitachi autosampler (Darmstadt, Germany), an HP 1050 HPLC pump (Waldbronn, Germany), a Jones Chroma-

tography 9730 column heater (Llanbradach, UK), an Applied Biosystems 783 variable UV detector (Ramsey, USA), and an L-5200 Merck-Hitachi fraction collector. Two 250 × 4.6 mm I.D. Zorbax ODS columns (Rockland Technologies, Chadds Ford, PA, USA), in tandem with a guard column (25 × 4 mm I.D. LiChrocart RP18) were used for retinoid isolation.

For micro-LC–MS the following apparatus was used: a CMA 200 Carnegie Medicine autosampler (Stockholm, Sweden); two Kontron 500 micro LC pumps (Zurich, Switzerland); and a Jones Chromatography 9730 column thermostat. For separation, a 250 × 0.32 mm I.D. micro column with a 1 × 0.25 mm I.D. guard column, both packed with 5 μm diol phase LC-Packings (Amsterdam, Netherlands), and an HP 5988 mass spectrometer (Palo Alto, USA) with an HP Chemstation (Palo Alto, USA) were used.

Procedure

A procedure for LC–MS analysis using negative ion chemical ionization and stable isotope dilution for the retinoic acids and their metabolites has been described previously [9,10]. We proceeded in a similar manner (Fig. 2) with the following changes:

(1) Each 1 ml of plasma was extracted three times with 2 ml of hexane.

(2) A solution of PFB–benzene (0.5:99.5, v/v) containing 150 mg of crown ether 18-crown-6 in 100 ml of benzene was prepared. After derivatization with this solution, the retinoic acid pentafluorobenzyl esters were separated from the 4-oxoretinoic acid pentafluorobenzyl esters by RP HPLC. An extra clean-up step was added to the RP-18 HPLC. A THF gradient (100% ACN for 18 min, 75% THF–25% ACN for 5 min, 100% ACN for 17 min) was used to prevent the collection of late eluting interfering peaks. The fractions of interest were then concentrated and dissolved in 10 μl of dry hexane.

(3) Only 200 nl of the solution were injected into the micro column, owing to capacity limitations. The mobile phases were hexane–toluene (95:5, v/v) with a flow-rate of 4 μl/min for the retinoic acid esters, and hexane–THF (97.5:2.5, v/v) with a flow-rate of 6 μl/min for the oxo metabolite esters. All concentration steps were carried out with a Univapo 150H vacuum centrifuge (Zivy, Basel, Switzerland).

(4) Ammonia was used as the reagent gas.

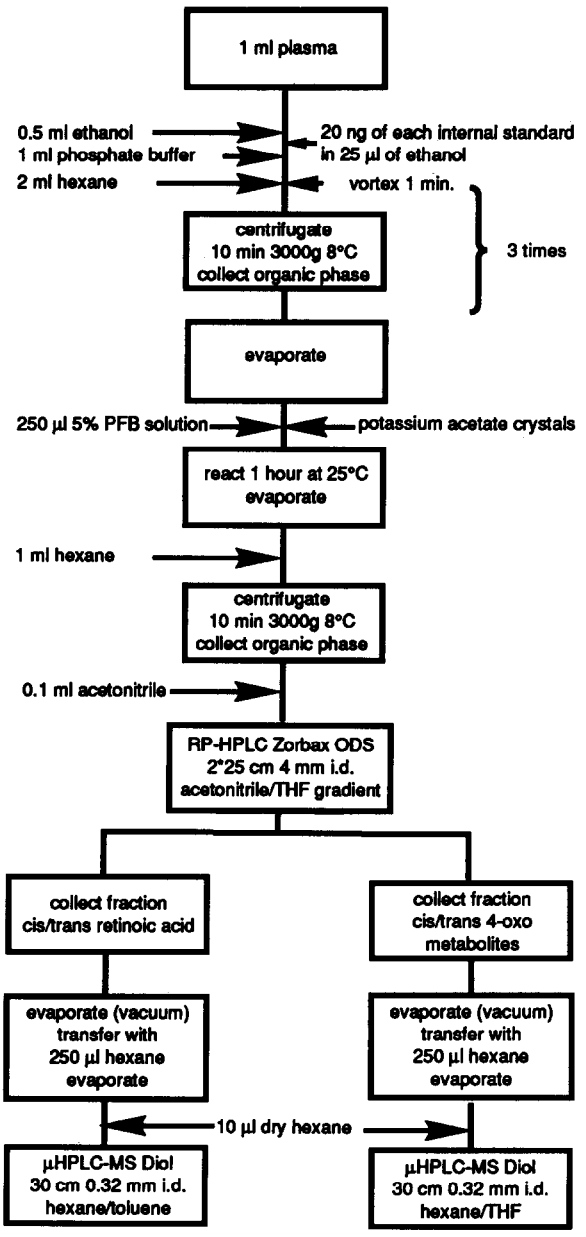

Fig. 2. Extraction and derivatization scheme for retinoic acids and the corresponding 4-oxo metabolites from human plasma.

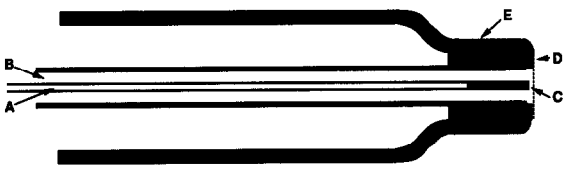

Fig. 3. Front of the LC–MS interface: A = fused-silica coupling capillary (50 μm I.D.); B = coaxial inlet for NH_3 reagent gas; C = frit made of waterglass; D = copper block for heat transfer from source block; E = stainless-steel rod entering through the direct inlet of the mass spectrometer.

LC–MS interface

The lower flow-rate for micro-HPLC did not allow the use of an interface of the kind described previously [9,10]. At first, we tried fused-silica capillaries with inner diameters of 10–100 μm that were externally heated. No stable conditions were obtained with this set-up. In contrast, using fused-silica capillaries with an I.D. of 50 μm ending with a frit consisting of porous waterglass, gave stable conditions without additional heat using a flow-rate of 2–10 μl/min. This type of restrictor is used for supercritical fluid chromatography and is commercially available from Dionex (Sunnyvale, USA). The frit was connected to the outlet capillary of the column with a zero dead volume connection. To avoid high column back pressures, the length of the frit was halved to *ca.* 5 mm. Fig. 3 shows a schematic diagram of the interface. The rod enters the vacuum manifold from the direct inlet side of the mass spectrometer.

RESULTS AND DISCUSSION

Attempts to separate all four compounds in one run were successful using a mobile phase containing ACN, but this mobile phase produced no negative ions. Furthermore, carbon black was deposited on the ion source, and the SFC-frit became clogged after a few hours of operation. Therefore, we used two mobile phase systems, as described previously [9,10]. The optimum ion source conditions were 200°C for the source temperature and 200 Pa for the ammonia (reagent gas) pressure.

Inter-assay statistics for all analytes were calculated from six series of calibration samples (Table I).

TABLE I

INTER-ASSAY PRECISION OF CALIBRATION SAMPLES

Sample	Spiked (ng/ml)	13-*cis* (n = 6)			All-*trans* (n = 6)		
		Mean (ng/ml)	S.D. (ng/ml)	Found (%)	Mean (ng/ml)	S.D. (ng/ml)	Found (%)
4-Oxoretinoic acid							
C0	0.0	−0.07	0.02		−0.04	0.04	
C1	0.3	0.35	0.03	116	0.34	0.01	115
C2	0.5	0.57	0.05	114	0.53	0.04	106
C3	1.0	0.98	0.07	98	0.94	0.07	94
C4	3.0	2.96	0.08	99	2.95	0.09	98
C5	7.0	7.10	0.27	101	7.05	0.29	101
C6	8.0	7.88	0.16	98	8.05	0.17	101
Retinoic acid							
C0	0.0	0.01	0.02		0.02	0.04	
C1	0.3	0.31	0.02	105	0.31	0.03	103
C2	0.5	0.50	0.02	100	0.48	0.03	97
C3	1.0	0.95	0.06	95	0.93	0.06	93
C4	3.0	2.93	0.10	98	2.98	0.12	99
C5	7.0	7.10	0.25	101	7.11	0.25	102
C6	8.0	8.10	0.16	101	8.07	0.18	101

TABLE II

TYPICAL CALIBRATION DATA FOR ALL-*TRANS*- AND 13-*CIS*-RETINOIC ACIDS AND 4-OXORETINOIC ACIDS CALCULATED USING WEIGHTED LINEAR LEAST-SQUARES FIT

Spiked (ng/ml)	All-*trans*			13-*Cis*		
	Found (ng/ml)	95% Confidence limit (± ng/ml)	Found (%)	Found (ng/ml)	95% Confidence limit (± ng/ml)	Found (%)
Retinoic acid						
0.00	−0.05	0.06		0.01	0.05	
0.30	0.34	0.06	114.6	0.30	0.04	99.8
0.50	0.48	0.06	95.3	0.52	0.04	104.0
1.00	1.10	0.06	109.6	0.95	0.04	95.0
3.00	2.95	0.13	98.3	2.99	0.10	99.6
7.00	6.94	0.32	99.1	6.82	0.24	97.4
8.00	7.99	0.37	99.9	8.26	0.29	103.3
4-Oxoretinoic acid						
0.00	0.04	0.07		0.05	0.05	
0.30	0.27	0.07	90.9	0.27	0.05	91.3
0.50	0.47	0.07	93.4	0.46	0.05	92.1
1.00	1.04	0.08	103.7	1.01	0.06	101.2
3.00	2.88	0.17	96.0	2.96	0.13	98.6
7.00	6.79	0.42	97.0	6.90	0.32	98.5
8.00	8.46	0.53	105.7	8.26	0.38	103.2

Table II shows typical calibration data in the range 0.3–8 ng/ml obtained for the retinoic acids and their 4-oxo metabolites. Typical ion chromatograms obtained from the plasma extract of a healthy volunteer are shown in Figs. 4 and 5. The traces at *m/z* 299.3 for the retinoic acids together with the ^{13}C internal standard at *m/z* 308.3 and, for the 4-oxo retinoic acids, at *m/z* 313.3 together with the ^{13}C internal standard at *m/z* 319.3 are shown. Endogenous plasma concentration ranges for the two isomers of retinoic acid and their respective 4-oxo metabolites were 1.19–1.99 ng/ml for 13-*cis*-retinoic acid, 1.4–1.76 ng/ml for all-*trans*-retinoic acid, 1.92–3.03 ng/ml for 13-*cis*-4-oxoretinoic acid, and below 0.3–0.7 ng/ml for the 4-oxo-all-*trans*-retinoic acid ($n = 8$). The number of theoretical plates necessary for resolution ranged from 60 000 to 70 000.

CONCLUSION

This assay measures 13-*cis*- and all-*trans*-retinoic acids and their corresponding 4-oxo metabolites in human plasma using normal phase micro-HPLC coupled in a straightforward way to a commercial quadrupole mass spectrometer. Baseline resolution was obtained only for the 4-oxo metabolites. However, 13-*cis*- and all-*trans*-retinoic acids were greater than 90% resolved. It was not possible to generate negative ions using a single mobile phase that could separate all four compounds, therefore two separate solvent systems were necessary. Compared with the HPLC methods using UV detection, HPLC–MS offers the advantage of increased sensitivity and selectivity. The assay was robust for more than 3000 injections over a period of 6 months.

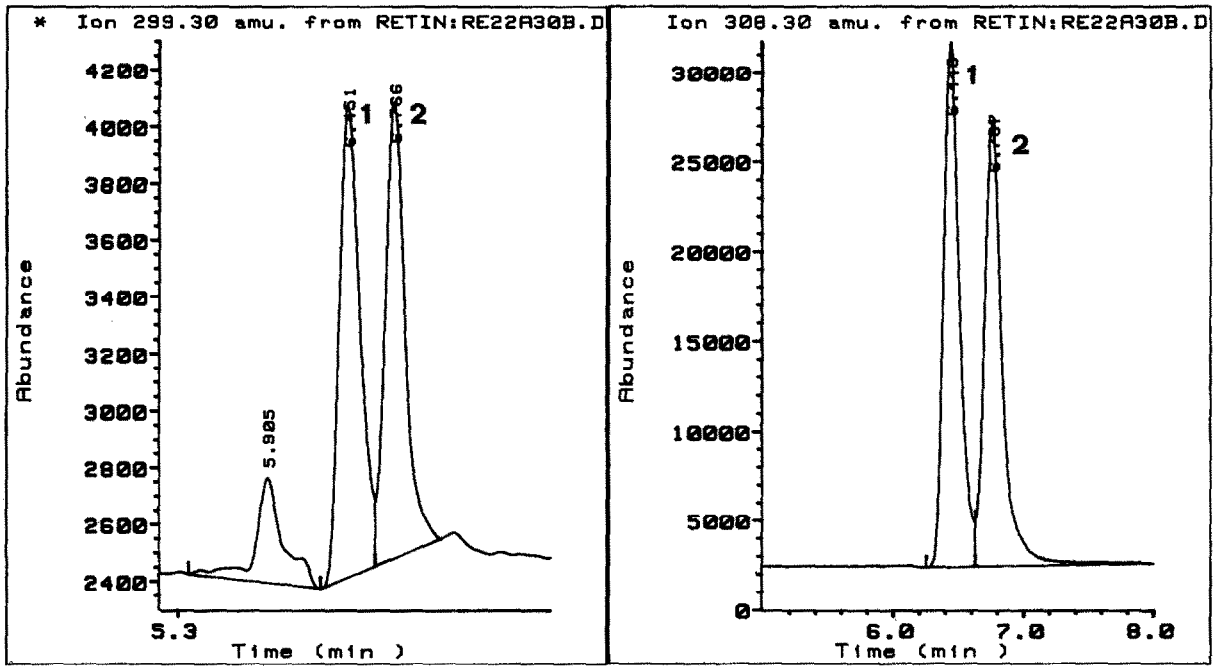

Fig. 4. Selected-ion chromatograms of endogenous 13-*cis*- (peak 1) and all-*trans* (peak 2) retinoic acids from the plasma extract of a healthy volunteer. (Left) Analytes monitoring *m/z* 299.3 (1.17 ng/ml for 13-*cis*- and 1.24 ng/ml for all-*trans*-retinoic acids). (Right) Internal standards monitoring *m/z* 308.3 (20 ng/ml each of labelled 13-*cis*- and all-*trans*-retinoic acids).

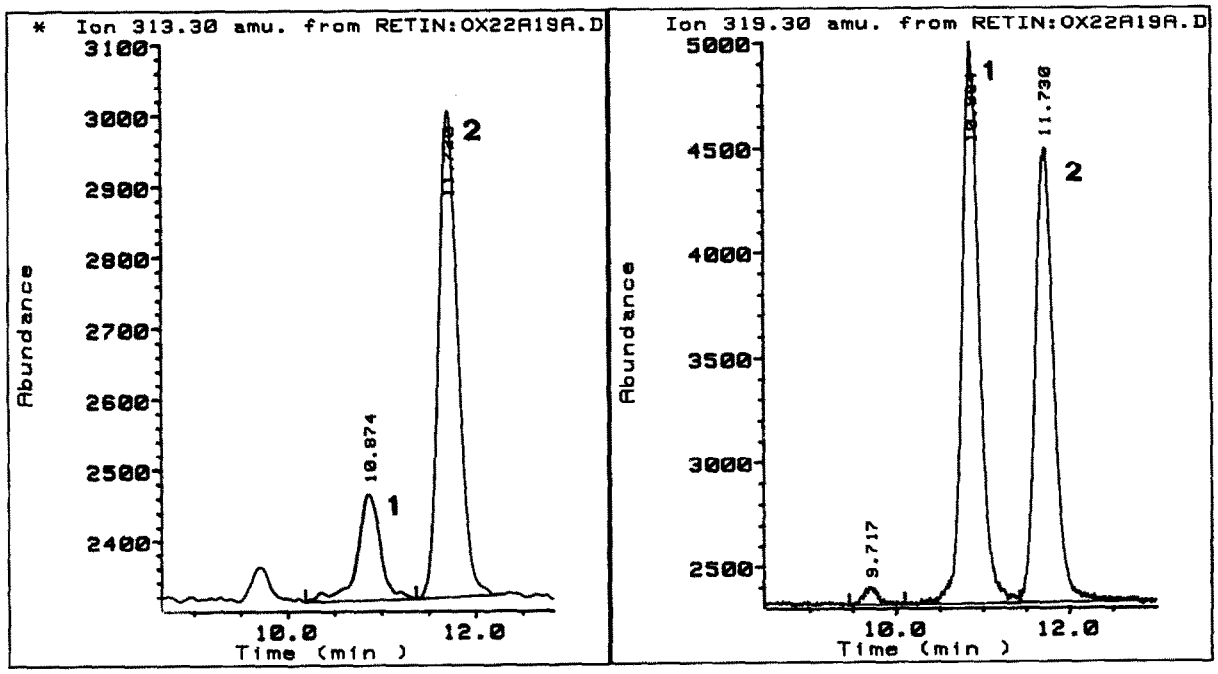

Fig. 5. Selected-ion chromatograms of the endogenous 4-oxo metabolites of all-*trans*- (peak 1) and 13-*cis*- (peak 2) retinoic acids from the plasma extract of a healthy volunteer. (Left) Analytes monitoring *m/z* 313.3 (0.79 ng/ml for all-*trans*-4-oxo- and 6.36 ng/ml for 13-*cis*-4-oxoretinoic acids). (Right) Internal standards monitoring *m/z* 319.3 (20 ng/ml each of labelled all-*trans*-4-oxo- and 13-*cis*-4-oxoretinoic acids).

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