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# Simultaneous determination of all-*trans*-, 13-*cis*-, 9-*cis*-retinoic acid and their 4-oxo-metabolites in plasma by high-performance liquid chromatography

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**Abstract**

A gradient reversed-phase high-performance liquid chromatographic technique is described for the easy separation and quantification of some retinoids; all-*trans*-retinoic acid, 13-*cis*-retinoic acid, 9-*cis*-retinoic acid and their corresponding 4-oxometabolites, in plasma. The method involved a diethyl ether–ethyl acetate (50:50, v/v) mixture extraction at pH 7 with acitretin and 13-*cis*-acitretin as internal standards. A Nova-Pak $C_{18}$ steel cartridge column was used. The mobile phase was methanol–acetonitrile (65:35, v/v) and 5% tetrahydrofuran (solvent A) and 2% aqueous acetic acid (solvent B) at 1 ml/min. The gradient composition was (only the percentages of solvent B are mentioned): I, 25% solvent B at the time of injection; II, 12% solvent B at 11 min until 30 min; III, 25% solvent B and maintenance of 25% solvent B for 10 min until a new injection. Total time between injections was 40 min. Detection was by absorbance at 350 nm. The precision calculated for plasma concentrations ranging from 2 to 250 ng/ml was better than 15% and the accuracy was less than 12%. The linearity of the method was in the range of 2 to 400 ng/ml of plasma. The limit of quantification was 2 ng/ml for each of the compounds. The HPLC method was applied to plasma specimens collected from animals receiving single dose administrations of all-*trans*-retinoic acid, 13-*cis*-retinoic acid and 9-*cis*-retinoic acid.

*Keywords:* Retinoic acids; 4-Oxoretinoic acids

## 1. Introduction

The retinoids are a large group of compounds that are structurally related to vitamin A (retinol). Retinoids have been shown to possess selective activities in proliferation, differentiation, keratinization, sebum production, inflammation, immune reaction and tumor prevention and therapy [1]. Tretinoin (all-*trans*-retinoic acid, all-*trans*-RA) and isotretinoin (13-*cis*-RA) are used in the treatment of severe cystic acne [2–4]. 9-*cis*-retinoic acid (9-*cis*-RA) was obtained by isomerisation of all-*trans*-RA [5]. In addition, 9-*cis*-RA binds to a subset of previously characterized nuclear receptors, known as retinoid X receptors (RXRs), in addition to the retinoic acid receptors (RARs), which are also known to be activated by all-*trans*-RA [6,7]. The pleiotropic biological effects of retinoids are thought to be mediated through these two families of receptors. RARs and RXRs are members of the superfamily of

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steroid–thyroid hormone receptors that act as ligand-dependent transcription factors. The main metabolites of these three retinoids in blood are their 4-oxo compounds, the simultaneous determination of all six compounds is desirable. Several methods have been developed for the determination of isotretinoin and/or tretinoin in blood, plasma or serum. These include normal-phase [8,9] and reversed-phase [10–12] HPLC methods. For the simultaneous determination of all-*trans*-RA or 13-*cis*-RA and its corresponding 4-oxometabolite, a gradient system is needed [13–19]. A few methods have been described for the determination of 9-*cis*-RA [20]. We have developed a gradient HPLC method allowing simultaneous quantification of all-*trans*-RA, 13-*cis*-RA and 9-*cis*-RA and their corresponding 4-oxometabolites from the same plasma sample, with a minimum quantity of solvents and reagents. The method was applied to a pharmacokinetic study in rabbits and rats receiving a single dose of all-*trans*-RA, 13-*cis*-RA and 9-*cis*-RA.

## 2. Experimental

### 2.1. Chemicals

All-*trans*-RA [Ro 01-5488, (2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, $M_r$=300.4], 13-*cis*-RA [Ro 04-3780, (2Z,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, $M_r$=300.4], 9-*cis*-RA [Ro 04-4079, (2E,4E,6Z,8E)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, $M_r$=300.4], all-*trans*-4-oxo-RA [Ro 12-4824, (2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethyl-3-oxo-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, $M_r$=314.5], 13-*cis*-4-oxo-RA [Ro 22-6595, (2Z,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethyl-3-oxo-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, $M_r$=314.5), 9-*cis*-4-oxo-RA [Ro 47-8078, (2E,4E,6Z,8E)-3,7-dimethyl-9-(2,6,6-trimethyl-3-oxo-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, $M_r$=314.5) and the internal standards acitretin [Ro 10-1670, (all-E)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid, $M_r$=326.44] and 13-*cis*-acitretin [Ro 13-7652, (2Z,4E,6E,8E)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid, $M_r$=326.44] were kindly provided by Hoffmann-La Roche (Basel, Switzerland).

Methanol, acetonitrile and tetrahydrofuran were of HPLC grade and were supplied, together with all other analytical grade reagents (glacial acetic acid, diethyl ether, ethyl acetate), from Carlo Erba (Milan, Italy). Buffers were obtained from Merck (Darmstadt, Germany). Water for HPLC was prepared by a Milli-Q-Water purification system.

### 2.2. Laboratory precaution

All handling of retinoids and biological samples was performed in a room with dim yellow light.

### 2.3. Standard solutions

Stock solutions of these compounds were prepared every three months by dissolving 10 mg of each compound in 10 ml of methanol, respectively. They were stored at −20°C in amber glass volumetric flasks, in a room with dim yellow light. Working solutions containing approximately 100 ng and 1000 ng/ml were obtained by sequential dilutions of the respective stock solutions in methanol every two weeks.

### 2.4. Blood collection and extraction procedures

Whole blood samples from animals were collected in vacutainers (BD Vacutainer tubes, 5 ml, containing 143 U.S.P. units lithium heparin, Becton-Dickinson, Meylan, France). The blood was immediately centrifuged and plasma was transferred to polypropylene tubes with frictionfit caps for freezing and storage at −20°C for less than three months.

In a 10-ml yellow amber tube, 25–100 μl of internal standard solutions (Ro 10-1670 and Ro 13-7652) were evaporated to near dryness under a stream of nitrogen. After addition of 0.2 to 1 ml of plasma and 0.1 ml of phosphate buffer, pH 7, (0.025 *M* $KH_2PO_4$ and 0.04 *M* $Na_2HPO_4.2H_2O$), the compounds were extracted for 5 min with 2 ml of a diethyl ether–ethyl acetate (50:50, v/v) mixture by vortex-mixing. After centrifugation at 2000 *g* for 10 min at 4°C, the organic phase was evaporated to dryness. The residue was dissolved in 30–100 μl of

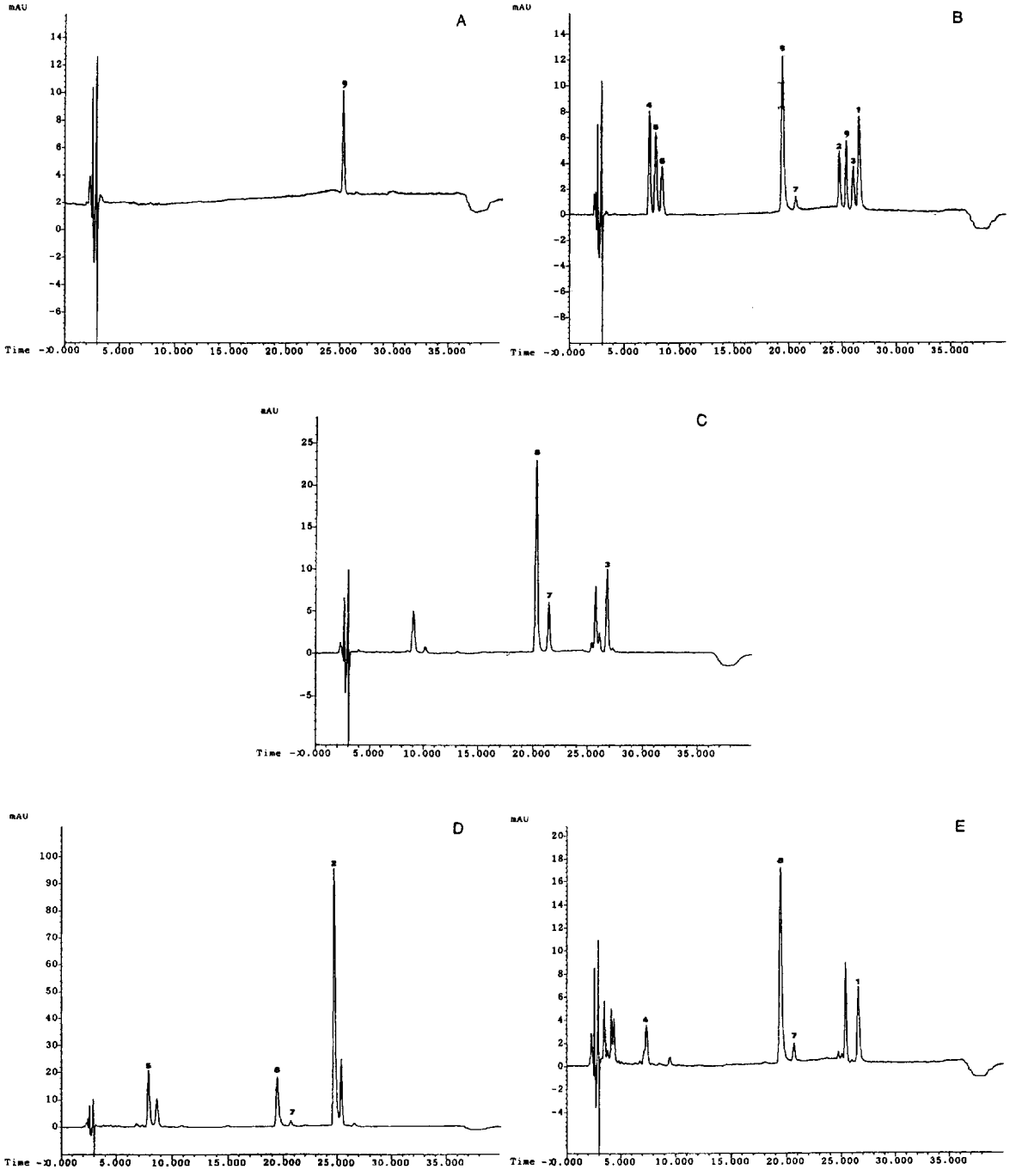


Fig. 1. Typical chromatograms obtained for: (A) control plasma (human plasma); (B) control plasma spiked with 100 ng/ml of all-*trans*-RA (1), 13-*cis*-RA (2), 9-*cis*-RA (3), all-*trans*-4-oxo-RA (4), 13-*cis*-4-oxo-RA (5), 9-*cis*-4-oxo-RA (6), 20 ng/ml of acitretin (7) and 200 ng/ml of 13-*cis*-acitretin (8); (C) 3 h after a single oral administration of 8 mg of 9-*cis*-RA to a rat; (D) 3 h after a single oral administration of 60 mg of 13-*cis*-RA to a rabbit; (E) 3 h after a single oral administration of 60 mg of all-*trans*-RA to a rabbit.

methanol and transferred to an injection vial for HPLC analysis.

### 2.5. Chromatographic conditions

HPLC analysis was performed on a HP 1090 apparatus (Hewlett-Packard) equipped with an autosampler and a variable-wavelength UV detector HP 1050. A Nova-Pak $C_{18}$ steel cartridge column (250× 4.6 mm I.D.) with 4 $\mu$m particles (Waters, Paris, France) was used at ambient temperature.

A binary gradient was formed from solvent A: methanol–acetonitrile (65:35, v/v) and 5% tetrahydrofuran and B: 2% aqueous acetic acid. The mobile phases were degassed by ultrasonic treatment prior to their use and by helium C with a flow-rate of 10 ml/min just prior to HPLC analysis and during HPLC analysis.

The gradient composition was (only the percentages of solvent B are mentioned): I, 25% solvent B at the time of injection; II, 12% solvent B at 11 min until 30 min; (the mobile phase changed linearly between 0 and 11 min), III, 25% solvent B at 30 min and maintenance of 25% solvent B for 10 min until a new injection. The automatic injector was programmed for an equilibration delay of 10 min and a run time of 26 min, which resulted in a 4-min hold at the final solvent conditions. Total time between injections was 40 min. The flow-rate was 1 ml/min. UV detection was carried out at 350 nm.

The sample containers for the automatic injector consisted of 0.3-ml glass microvials (Hewlett-Packard) inserted into black sleeves. The microvials were capped with open screw caps fitted with self-sealing septa. Microvials and black sleeves were then inserted into the black microvial tray. A 25-$\mu$l aliquot of each sample was injected.

### 2.6. Procedures for the evaluation of linearity and limit of quantification

Standard curves with control plasma spiked by the six compounds and internal standard were established for each analysis in the range of 2 to 400 ng/ml.

### 2.7. Calculations

The ratios of the peak heights of all-*trans*-4-oxo-RA, 13-*cis*-4-oxo-RA, 9-*cis*-4-oxo-RA, 13-*cis*-RA, 9-*cis*-RA and all-*trans*-RA to the peak heights of the internal standards (acitretin, 13-*cis*-acitretin) were determined from the chromatograms. Acitretin was used at a high concentration and 13-*cis*-acitretin at a lower concentration because the range between concentrations of analytes and metabolites was too large, so we need two calibration curves for good calculations. Peak-height ratios of analytes to internal standards were computed by means of the Vectra data system QS/16S. Data on plasma concentrations of analyzed compounds were obtained from least-squares linear regression curves, established daily from seven calibration points.

## 3. Results

### 3.1. Chromatograms

Under the described conditions, retention times for all-*trans*-4-oxo-RA, 13-*cis*-4-oxo-RA, 9-*cis*-4-oxo-RA, the internal standards 13-*cis*-acitretin and acitretin, 13-*cis*-RA, 9-*cis*-RA and all-*trans*-RA were 7.4, 8.1, 8.7, 19.8, 21, 24.9, 26.2 and 26.7 min, respectively. The retention times without the gradient elution were the same for the 4-oxo-metabolites but were too long (>1 h) for the native compounds.

Fig. 1 shows typical chromatograms obtained under the described analytical conditions for (A) control plasma; (B) control plasma spiked with 13-*cis*-RA (peak 2), 9-*cis*-RA (peak 3), all-*trans*-RA (peak 1), 13-*cis*-4-oxo-RA (peak 5), 9-*cis*-4-oxo-RA (peak 6), all-*trans*-4-oxo-RA (peak 4) and the internal standards [acitretin (peak 7) and 13-*cis*-acitretin (peak 8)]; (C) 3 h after a single oral administration of 8 mg of 9-*cis*-RA to a rat; (D) 3 h after a single oral administration of 60 mg of 13-*cis*-RA to a rabbit and (E) 3 h after a single oral administration of 60 mg of all-*trans*-RA to the same rabbit. Other peaks were observed in some chromatograms, possibly other metabolites of the administered compounds. A satisfactory separation was achieved from endogenous plasma components,

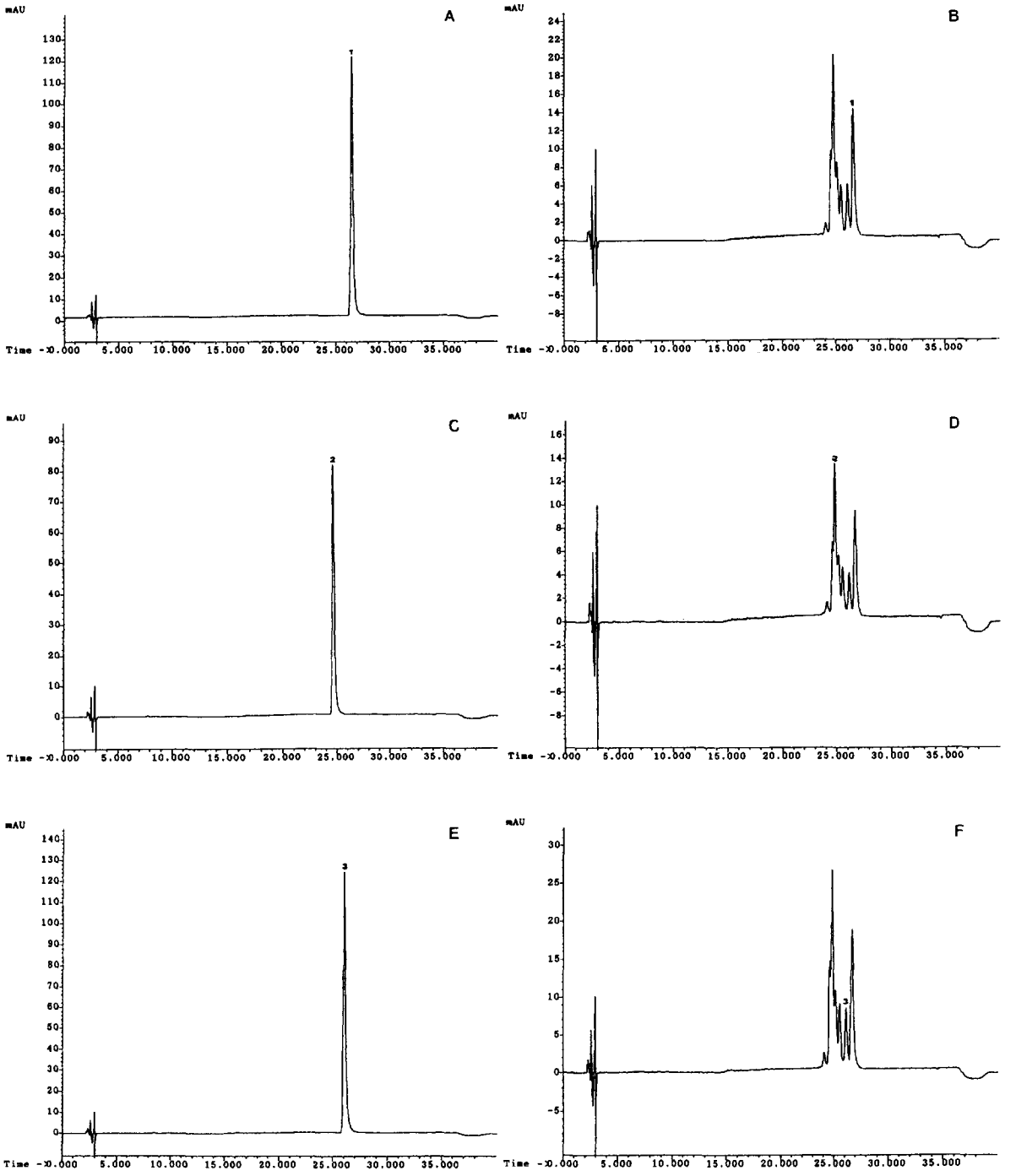


Fig. 2. (a) Stability of the working solutions after 24 h for (A) all-*trans*-RA under yellow light; (B) all-*trans*-RA under natural light; (C) 13-*cis*-RA under yellow light; (D) 13-*cis*-RA under natural light; (E) 9-*cis*-RA under yellow light and (F) 9-*cis*-RA under natural light. (b) Stability of the working solutions after 24 h for (A) all-*trans*-4-oxo-RA under yellow light; (B) all-*trans*-4-oxo-RA under natural light; (C) 13-*cis*-4-oxo-RA under yellow light; (D) 4-oxo-13-*cis*-RA under natural light; (E) 4-oxo-9-*cis*-RA under yellow light and (F) 4-oxo-9-*cis*-RA under natural light.

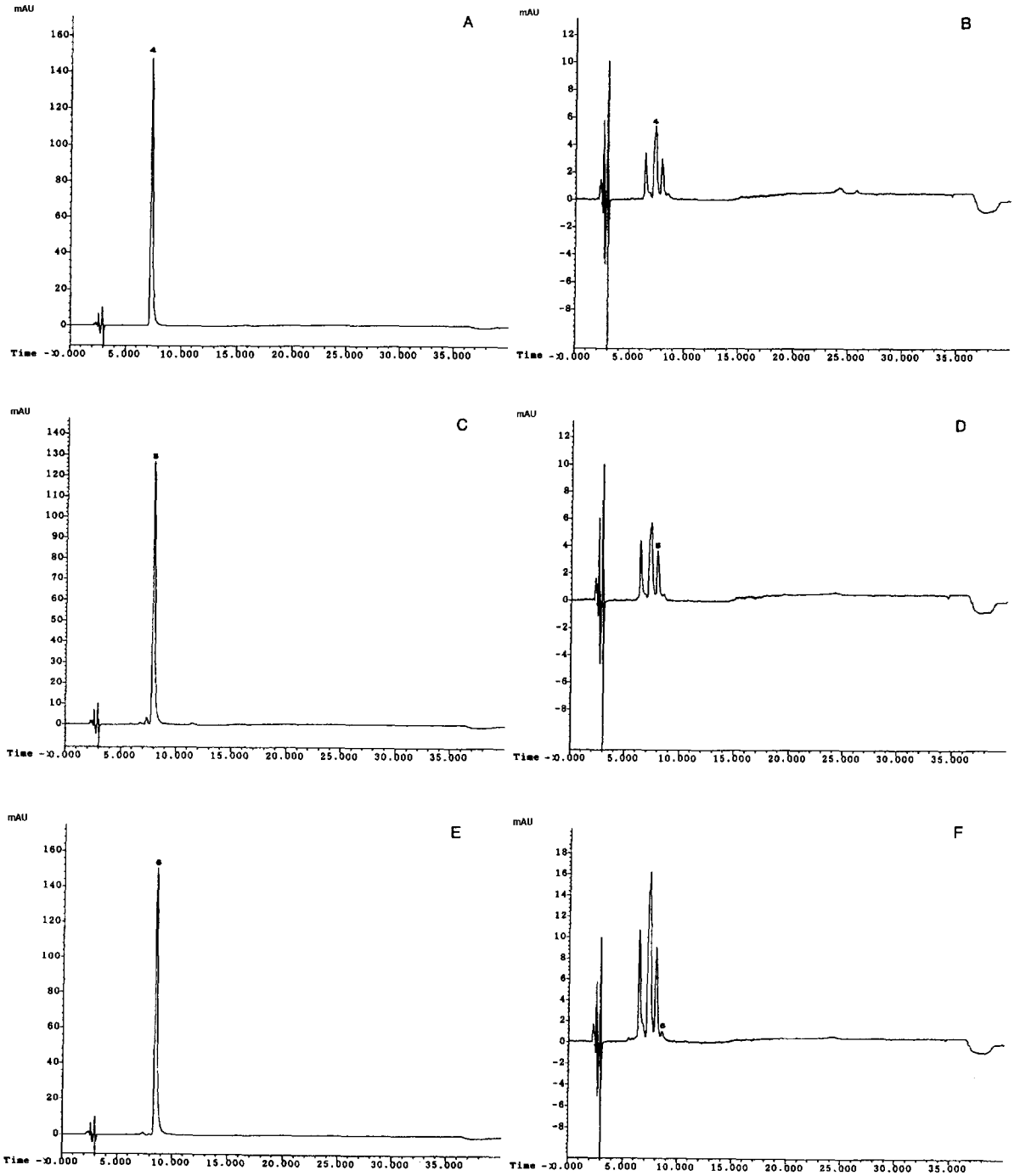


Fig. 2. (*continued*)

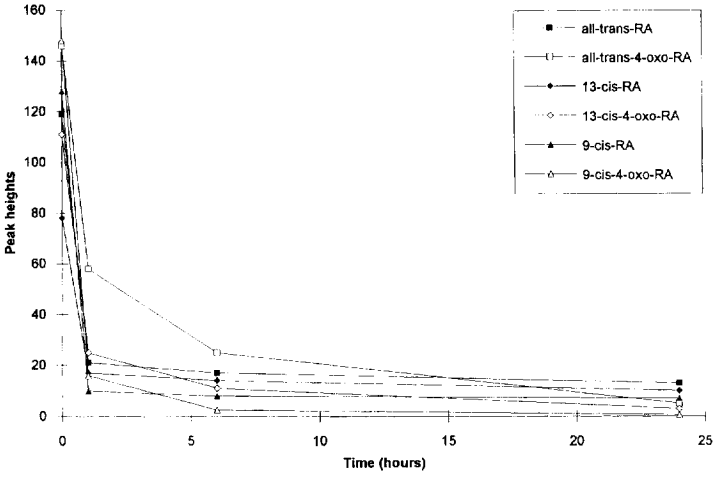


Fig. 3. Isomerisation or degradation curves of the six compounds under natural light.

which seem to be vitamin A. (Fig. 1A–B, peak 9). Chromatograms of human, rat and rabbit were similar.

### 3.2. Stability

#### 3.2.1. Working solutions

The stability of the working solutions of these compounds was tested under natural light and yellow light for periods of up to 24 h at ambient temperature. The analytes were stable under yellow light, but they were very unstable under natural light conditions (Fig. 2a, Fig. 2b). Isomerisation or degradation of the compounds under natural light was very rapid, the peak heights of these compounds at 1 h for initial time were below 50% (Fig. 3). 9-*cis*-RA and 9-*cis*-4-oxo-RA were the most rapidly degraded. After 24 h under natural light, the values of the peak heights of these compounds were below 10%.

All isomers tested individually (all-*trans*-RA, 13-*cis*-RA, 9-*cis*-RA), produced three peaks corresponding to the three isomers. The same results were observed for 4-oxometabolites. Each compound was analyzed in duplicate per time point.

Control plasmas, separately spiked with the investigated compounds at two concentrations (50 and 100 ng/ml), were stored successively for three and seven days at 4°C, and for 7, 14, 30, 60 and 90 days at −20°C. For each storage duration and temperature, these spiked plasmas were analyzed in duplicate, using a calibration curve established daily. For an added concentration of 50 ng/ml, the means of the values obtained after different storage times at −20°C were 51.2 ng/ml of all-*trans*-RA, 50.5 ng/ml of 13-*cis* RA, 49.8 ng/ml of 9-*cis*-RA, 48.8 ng/ml of all-*trans*-4-oxo-RA, 49.2 ng/ml of 13-*cis*-4-oxo-RA and 49.9 ng/ml of 9-*cis*-4-oxo-RA. For an added concentration of 100 ng/ml, the means of the values obtained were 100 ng/ml of all-*trans*-RA, 97.9 ng/ml of 13-*cis*-RA, 100 ng/ml of 9-*cis*-RA, 94.4 ng/ml of all-*trans*-4-oxo-RA, 95.9 ng/ml of 13-*cis*-4-oxo-RA and 99.5 ng/ml of 9-*cis*-4-oxo-RA. Whatever the added concentrations or the different storage times at 4°C or at −20°C, the inaccuracy was less than 11%. No significant differences ($p=0.05$) were observed for these six compounds under the storage conditions investigated.

#### 3.2.2. Precision and accuracy

The precision (given by the relative standard deviation) and the inaccuracy (defined by the difference between found and expected concentrations) were calculated for plasma concentrations of these compounds ranging from 2 to 250 ng/ml. The results were acceptable within these concentration ranges;

Table 1
Intra-assay precision and accuracy for plasma determination ($n=8$ in all cases)

| Added concentration (ng/ml) | Obtained concentration (ng/ml) | Coefficient of variation (%) | Bias (%) |
|---|---|---|---|
| *All-trans-RA* | | | |
| 2 | 2.2 | 15.0 | 9.5 |
| 5 | 5.6 | 8.3 | 12.0 |
| 10 | 10.0 | 10.3 | 0 |
| 25 | 26.3 | 5.8 | 5.2 |
| 50 | 49.6 | 6.0 | −0.8 |
| 100 | 104.7 | 3.2 | 4.7 |
| 250 | 246.6 | 6.5 | −1.3 |
| *13-cis-RA* | | | |
| 2 | 2.2 | 11.8 | 10.0 |
| 5 | 5.3 | 10.7 | 6.0 |
| 10 | 10.5 | 10.4 | 5.0 |
| 25 | 25.4 | 3.0 | 1.6 |
| 50 | 52.9 | 5.6 | 5.8 |
| 100 | 109.0 | 7.2 | 9.0 |
| 250 | 251.2 | 6.3 | 0.5 |
| *9-cis-RA* | | | |
| 2 | 2.1 | 15.0 | 5.0 |
| 5 | 5.5 | 8.0 | 10.0 |
| 10 | 10.5 | 3.4 | 5.0 |
| 25 | 26.8 | 3.0 | 7.2 |
| 50 | 49.0 | 5.0 | −2.0 |
| 100 | 94.0 | 7.0 | −6.0 |
| 250 | 257.0 | 7.8 | 2.8 |
| *all-trans-4-oxo-RA* | | | |
| 2 | 2.0 | 10.9 | 1.5 |
| 5 | 4.8 | 9.4 | −4.0 |
| 10 | 10.6 | 8.5 | 6.0 |
| 25 | 23.8 | 3.1 | −4.8 |
| 50 | 50.8 | 2.5 | 1.6 |
| 100 | 108.0 | 3.8 | 8.0 |
| 250 | 237.0 | 8.5 | −5.2 |
| *13-cis-4-oxo-RA* | | | |
| 2 | 2.2 | 9.3 | 10.0 |
| 5 | 5.3 | 8.5 | 6.0 |
| 10 | 9.9 | 1.8 | −1.0 |
| 25 | 24.0 | 3.6 | 4.0 |
| 50 | 53.5 | 5.6 | 7.0 |
| 100 | 101.5 | 3.3 | 1.5 |
| 250 | 254.0 | 8.5 | 1.6 |
| *9-cis-4-oxo-RA* | | | |
| 2 | 2.1 | 10.7 | 5.5 |
| 5 | 4.6 | 8.1 | −8.0 |
| 10 | 10.1 | 9.4 | 1.0 |
| 25 | 24.3 | 4.2 | −2.8 |
| 50 | 51.9 | 6.6 | 3.8 |
| 100 | 102.9 | 3.2 | 2.9 |
| 250 | 240.0 | 7.9 | −4.0 |

Table 2
Extraction recovery of all-*trans*-RA, 13-*cis*-RA, 9-*cis*-RA, all-*trans*-4-oxo-RA, 13-*cis*-4-oxo-RA and 9-*cis*-4-oxo-RA from plasma ($n=7$)

| Concentration (ng/ml) | Extraction recovery (%) | Coefficient of variation (%) |
|---|---|---|
| *All-trans-RA* | | |
| 10 | 83 | 11.6 |
| 25 | 82 | 4.2 |
| 50 | 80 | 5.3 |
| 100 | 75 | 3.1 |
| *13-cis-RA* | | |
| 10 | 80 | 8.4 |
| 25 | 77 | 11.7 |
| 50 | 72 | 5.9 |
| 100 | 82 | 3.1 |
| *9-cis-RA* | | |
| 10 | 79 | 4.9 |
| 25 | 80 | 5.9 |
| 50 | 76 | 4.0 |
| 100 | 83 | 4.6 |
| *all-trans-4-oxo-RA* | | |
| 10 | 76 | 9.7 |
| 25 | 86 | 5.9 |
| 50 | 74 | 7.4 |
| 100 | 78 | 7.5 |
| *13-cis-4-oxo-RA* | | |
| 10 | 74 | 11.8 |
| 25 | 76 | 8.1 |
| 50 | 73 | 8.8 |
| 100 | 76 | 7.3 |
| *9-cis-4-oxo-RA* | | |
| 10 | 76 | 7.3 |
| 25 | 82 | 9.7 |
| 50 | 68 | 7.4 |
| 100 | 72 | 5.7 |

the precision was better than 15% and the bias was less than 12% (Table 1).

### 3.3. Linearity

The linearity of the method was checked for concentrations of compounds (all-*trans*-RA, 13-*cis*-RA, 9-*cis*-RA and their 4-oxometabolites) in the range of 2 to 400 ng/ml of plasma. The correlation coefficients were in the range 0.994 to 0.998 and the intercepts of the calibration curves did not differ significantly from zero.

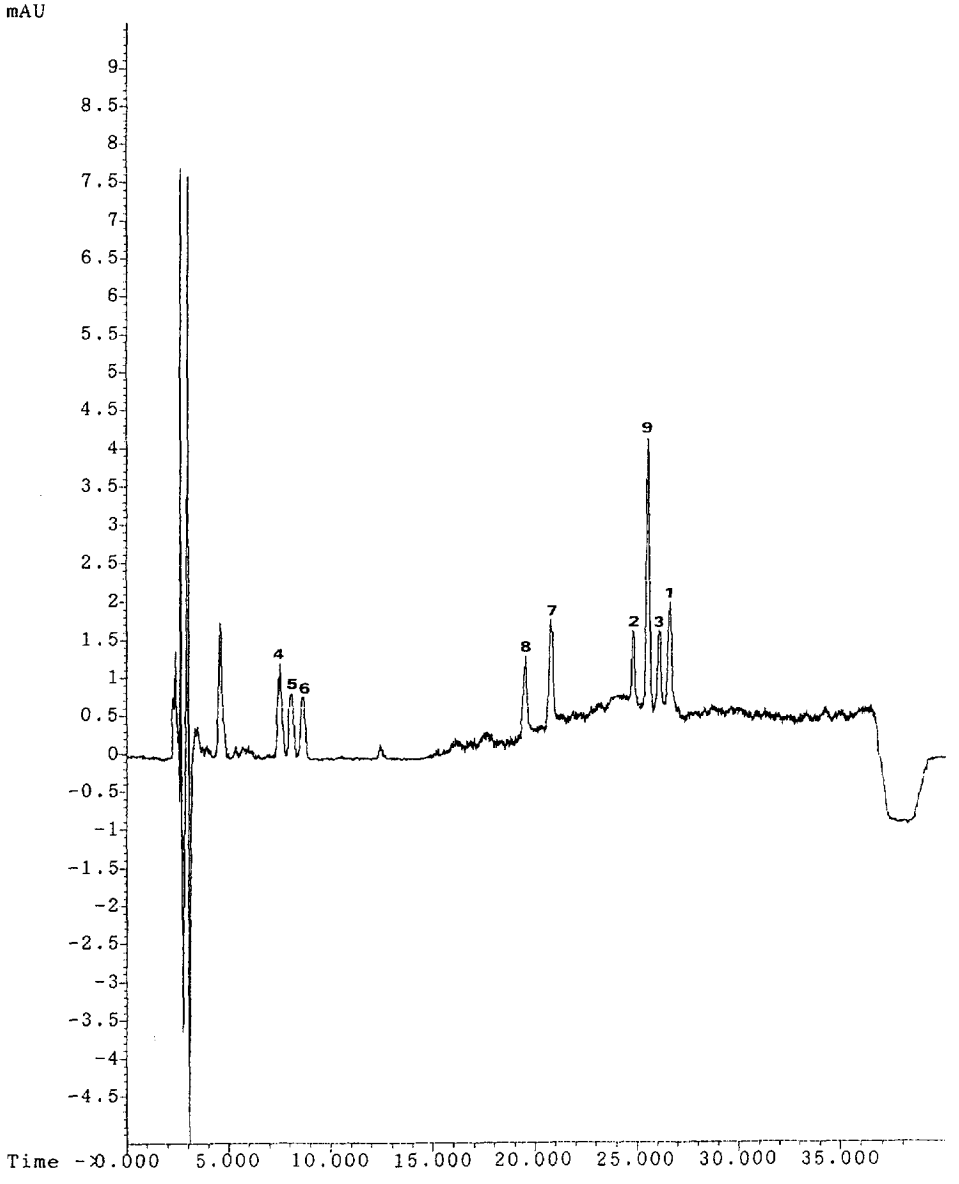


Fig. 4. Control plasma spiked with 2 ng/ml of the eight compounds.

### 3.4. Recovery

Each concentration was determined in replicate ($n=7$), according to the described method. Recovery was calculated by comparing the measured peak height of these compounds of spiked serum to those of the standard solutions.

Recovery from human plasma was in the range of 68–86%, regardless of the concentration of the compound (Table 2).

### 3.5. Limit of quantification

The limit of quantification was estimated to be 2 ng/ml of plasma for all compounds (Fig. 4).

Near this limit, intra-assay reproducibility and

Table 3
Inter-assay precision and accuracy for the limit of quantification ($n=7$ in all cases)

| | Mean concentration obtained (ng/ml) | Coefficient of variation (%) | Bias (%) |
|---|---|---|---|
| All-*trans*-RA | 2.2 | 19.4 | 10 |
| 13-*cis*-RA | 2.46 | 17.5 | 23 |
| 9-*cis*-RA | 2.23 | 21 | 11 |
| All-*trans*-4-oxo-RA | 1.86 | 12 | 7 |
| 13-*cis*-4-oxo-RA | 2.22 | 16 | 11 |
| 9-*cis*-4-oxo-RA | 2.35 | 14.3 | 17 |

accuracy were 15%. Inter-assay precision and accuracy were in the range of 10–23% for all compounds (Table 3).

### 3.6. Selectivity

Administration of retinoids to patients is frequently associated with other drugs such as psychotropic, antibiotic or other.

Some forty drugs (alprazolam, bromazepam, clonazepam, diazepam, flunitrazepam, lorazepam, midazolam, nitrazepam, nordiazepam, oxazepam, amitriptyline, carbamazepine, chlorpromazine, haloperidol, meprobamate, phenytoin, amikacin, amphotericin B, ceftriaxone, erythromycin, netilmicin, nystatin, acyclovir, azidothymidine, ketoconazole, metronidazole, miconazole, sulconazole, prednisone, prednisolone, methylprednisolone, nifedipine, atenolol, thiopental, theophylline, caffeine, cimetidine, dextromethorphan, paracetamol, acetylsalicylic acid) were tested for possible interference. No interference was observed for any of these drugs. For endogenous plasma components, the specificity was satisfactory (Fig. 1).

## 4. Discussion

When we described these conditions of analysis, only a few methods were described for 9-*cis*-RA. Marchetti et al. [20] developed a method for analysing 9-*cis*-RA, all-*trans*-RA and 13-*cis*-RA. The method described by Collins et al. [21] used two detection wavelengths (340 and 386 nm). In the method we have described, we used a wavelength of 350 nm.

Several chromatographic conditions were tested for the simultaneous determination of the compounds. Solvents (methanol, acetonitrile, water) and reagents (ammonium acetate, phosphate buffer, triethylamine) in different ratios for isocratic or gradient elution and with $\mu$Bondapack $C_{18}$, Nucleosil $C_{18}$ and $C_{18}$ Nova-pak as the stationary phase were tested. Good resolution was obtained under the analytical conditions described. The different conditions we tested could not separate the 4-oxo-metabolites. In the described method, we obtained a good separation for all compounds. The retention times

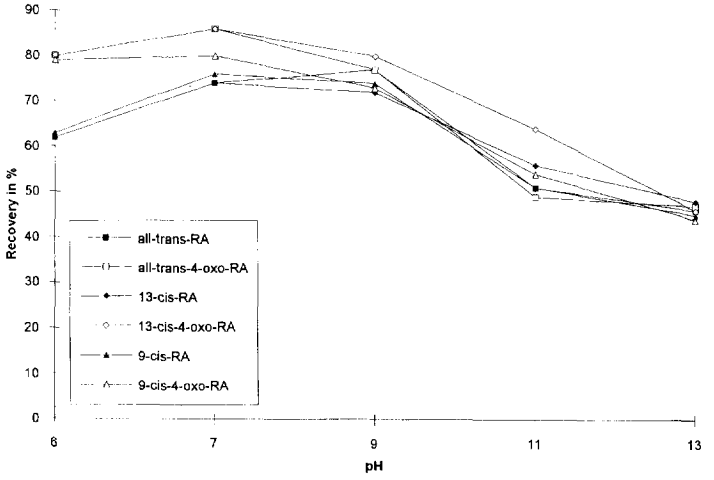


Fig. 5. Influence of pH on extraction efficiency.

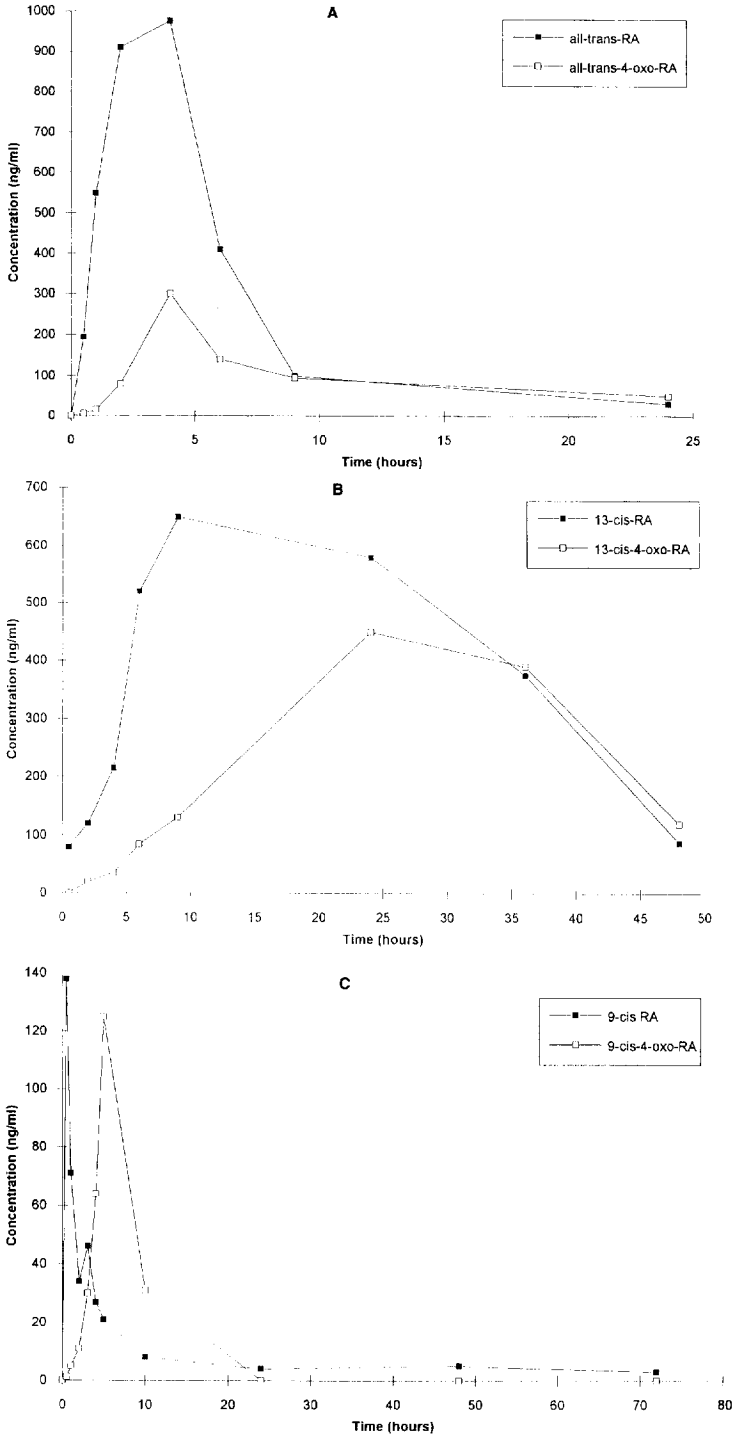


Fig. 6. (A) Time course of plasma concentration of all-*trans*-RA and 4-oxo-all-*trans*-RA after a single oral dose of 60 mg of all-*trans*-RA to a rabbit. (B) Time course of plasma concentration of 13-*cis*-RA and 4-oxo-13-*cis*-RA after a single oral dose of 60 mg of 13-*cis*-RA to a rabbit. (C) Time course of plasma concentration of 9-*cis*-RA and 4-oxo-9-*cis*-RA after a single oral dose of 8 mg of 9-*cis*-RA to a rat.

without the elution gradient were too long for the native compounds.

All-*trans*-RA, 13-*cis*-RA, 9-*cis*-RA and their respective oxometabolites were extracted in neutral medium (pH 7). We tested recovery with acidic, neutral and alkaline medium over a large range of pH values: pH 1 (citrate buffer), pH 3 (citrate buffer), pH 4 (citrate buffer), pH 5 (citrate buffer), pH 6 (citrate buffer), pH 7 (phosphate buffer), pH 9 (borate buffer), pH 11 (borate buffer) and pH 13 (borate buffer). We added 100 $\mu$l of buffer to the medium, each analysis was performed in duplicate for each pH value. We analysed the results qualitatively by comparing the peak heights of each compound with two gauges. The results showed that extraction recoveries in acidic medium were too low. Extraction effiency was improved with borate buffer (pH 9) for unchanged compounds and with phosphate buffer (pH 7) for all compounds (Fig. 5). We chose pH 7 because of the good quality of the chromatograms.

## 5. Application

As an example, the proposed HPLC method was applied to the determination of all-*trans*-RA, 13-*cis*-RA and 9-*cis*-RA and their respective oxometabolites in plasma from rabbit and rat, administered orally as a single dose of 60 mg of all-*trans*-RA or 13-*cis*-RA and 8 mg of 9-*cis*-RA in a 5% arabic gum solution. Time courses of the plasma concentration of all-*trans*-RA, 13-*cis*-RA and 4-oxometabolites after the single dose of all-*trans*-RA and of 13-*cis*-RA from the same rabbit are shown in Fig. 6A, B and the 9-*cis*-RA results, following administration of 9-*cis*-RA, are shown in Fig. 6C. We chose a rabbit without pharmacokinetic characteristics because the results demonstrated the variability in intestinal absorption and in metabolism of the compounds and the presence of an entero-hepatic cycle. An extensive pharmacokinetic treatment of the results will be the subject of a further publication.

This proposed HPLC method can be applied to the determination of these compounds in other matrices (skin, bile, microsomes) with few modifications.

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