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# Simple reversed-phase high-performance liquid chromatographic method for 13-*cis*-retinoic acid in serum

R. Rao Gadde* and Frederick W. Burton

*Bristol-Myers Squibb Company, Pharmaceutical Research Institute, 100 Forest Avenue, Buffalo, NY 14213 (USA)*

ABSTRACT

An isocratic reversed-phase high-performance liquid chromatographic method for the analysis of 13-*cis*-retinoic acid in serum is developed. Sample preparation includes deproteination with acetonitrile–perchloric acid–acetic acid followed by centrifugation. 9-Methylanthracene is used as the internal standard. Chromatographic separation is achieved on a $C_{18}$ column (Zorbax) using an acetonitrile–aqueous 0.5% acetic acid (85:15, v/v) eluent containing 0.05% (w/v) sodium hexanesulfonate. The limit of detection is 12 ng/ml in serum, using 0.5 ml samples. Quantitative recoveries and excellent intra-day and inter-day precision are reported.

## INTRODUCTION

13-*cis*-Retinoic acid (CRA) (Fig. 1) is a very effective drug in the treatment of severe, recalcitrant cystic acne [1]. It is also under study for the treatment of several keratinizing disorders (psoriasis, ichthyosis, etc.) and epithelial cancers. The pharmacology, clinical pharmacokinetics and therapeutic efficacy of CRA have been reviewed [1–3]. It has been extensively studied in animals and humans to evaluate the safety and efficacy of this drug. Different chromatographic methods used to monitor the retinoids, including CRA, in biological fluids and tissues have been discussed in a recent review [4].

CRA, like most retinoids, is very sensitive to light and oxidation [4–8]. Multiple degradation products, isomers as well as oxidation products, were reported [9,10]. Extreme precautions to protect CRA from white light to minimize exposure to oxygen by inert gas purge, and to maintain storage of samples at low temperatures (−17°C to −70°C) are recommended in handling CRA and its solutions [4–8,11]. The assay method for biological fluids analysis should therefore be simple with few sample handling manipulations, yet specific to resolve CRA from its metabolite, degradation products and matrix components. However, most of the published methods are cumbersome and complex for routine use.

Lengthy sample preparation schemes including extractions with an organic solvent, evaporation of solvent and reconstitution prior to high-performance liquid chromatographic (HPLC) analysis were used in several published procedures [11–14]. Lyo-

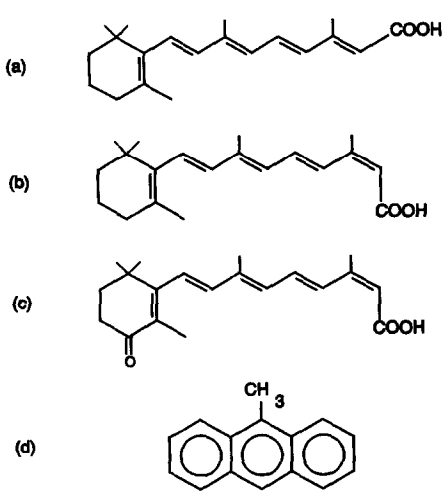


Fig. 1. Structural configurations. a = All-*trans*-retinoic acid, b = 13-*cis*-retinoic acid, c = 4-oxo-13-*cis*-retinoic acid and d = 9-methylanthracene.

philization of the sample before extraction is required in other methods [15,16]. Solvent gradient programming is required in some methods [7,13,14,17,18]. Both column switching and gradient elution were used in one fully automated method [19,20]. The present report describes an isocratic reversed-phase HPLC method for CRA in human serum which is precise, accurate and sensitive and requires only minimal sample preparation.

## EXPERIMENTAL

### *Apparatus*

A Waters Assoc. (Milford, MA, USA) Model 204 liquid chromatograph was used. It was equipped with Model 6000A low-volume displacement pump, a Model U6K universal injector, Model 710A intelligent sample processor (WISP), Model 440 absorbance detector (365 nm) and Zorbax 5 μm ODS column (25 cm × 4.6 mm I.D., DuPont, Wilmington, DE, USA). The chromatograms were recorded using an OmniScribe B5117-1 recorder (Houston Instruments, Austin, TX, USA). A Hewlett-Packard Model 3352D Laboratory Data System was used for peak height determination and data analysis.

The HPLC mobile phase used was acetonitrile–aqueous 0.5% acetic acid (85:15, v/v) containing 0.05% (w/v) sodium hexanesulfonate. The flow-rate was 2 ml/min. The injection volume for samples and standards was 50 μl. The detection wavelength was 365 nm.

### *Reagents and chemicals*

CRA was obtained from BASF Wyandotte (Wyandotte, MI, USA). 4-Oxo-13-*cis*-retinoic acid (OCRA) was provided by Midwest Research Institute (Kansas City, MO, USA). All-*trans*-retinoic acid (TRA) and sodium hexanesulfonate were acquired from Eastman Kodak (Rochester, NY, USA). ACS grade methanol and glacial acetic acid, HPLC grade acetonitrile and also certified reagent perchloric acid solution (0.1 *M* in acetic acid) were purchased from Fisher Scientific (Rochester, NY, USA). Dimethyldichlorosilane was obtained from Pierce (Rockford, IL, USA), 9-methylanthracene from Aldrich (Milwaukee, WI, USA) and human serum from Interstate Blood Bank (Philadelphia, PA, USA). Reagent grade water from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used in making the HPLC mobile phase.

### *General procedures*

All solvents, serum and mobile phase were deaerated before use by sonication under vacuum. The headspace over the solutions was flushed with argon. All CRA solutions were also protected from UV light by using low actinic glassware and peforming the solution transfers in yellow light only. The 4-ml vials (Sun Brokers, Wilmington, NC, USA) used for sample preparation and also as vials for automatic injector (WISP) are silanized before use, with dimethyldichlorosilane.

### *Preparation of standards*

A CRA stock standard solution (0.5 mg/ml) was prepared in methanol and standardized periodically using a combination of spectrophotometric and liquid chromatographic methods described earlier [5]. When not in use, it was stored in a refrigerator (5°C). Dilute CRA solution (25 μg/ml) in methanol for use in preparing calibration standards were prepared fresh by diluting the stock solution. The stock solution of internal standard 9-methylanthracene (50 μg/ml nominal concentration) was also prepared in methanol and diluted further as needed.

Six different spiking standard solutions were prepared from the above dilute CRA solution and the methyl anthracene solution, to contain 25–1000 ng/ml of CRA and 6 μg/ml of 9-methylanthracene. Serum CRA standards were prepared by pipeting 0.5 ml of pooled serum, 100 μl of the spiking standard solution, 1.5 ml of acetonitrile and 100 μl of 0.1 *M* perchloric acid solution. The head space in vials was flushed with argon, and the vials were capped with self-seal septa and mixed over a vortex mixer. The vials were then centrifuged at 10°C until the solution was clear (approximately 5 min, 300 *g*) and transferred to an automatic injector for HPLC analysis.

The spiked serum standards prepared as above represent CRA concentrations in the range of 50–2000 ng CRA/ml in serum. These standards were used in the routine analysis of serum and plasma samples. Standards out of this concentration range were prepared and used as needed in evaluating the method.

*Preparation of samples*

A 0.5-ml aliquot of plasma or serum sample in a 4-ml vial was spiked with 100 μl of dilute internal standard solution (6 μg/ml) and then treated with 1.5 ml of acetonitrile and 100 μl of 0.1 *M* perchloric acid solution. The headspace over the mixture was then flushed with argon, and the vial was capped, vortexed, centrifuged and loaded into an HPLC automatic injector for analysis.

## RESULTS AND DISCUSSION

*Chromatography*

For the analysis of CRA and its metabolite in biological fluids and tissues, reversed-phase HPLC methods using octadecylsilane columns have been the methods of choice. Different sample preparation procedures, eluent systems and also octadecylsilane columns from different manufacturers have been used to advantage. Most of these methods reviewed recently [4] have either used lengthy sample preparation schemes (lyophilization, solid phase or liquid–liquid extractions), gradient elution systems or both. Our efforts were focussed on simplifying the sample preparation process and developing a sensitive and specific method for CRA and its metabolites.

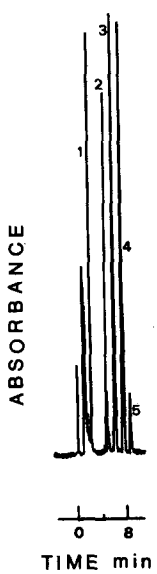


Fig. 2. Chromatogram of spiked serum sample showing separation of OCRA (1), 9-methylanthracene (2), CRA (3), TRA (4) and endogenous retinol (5). Detection wavelength 365 nm. See text for sample preparation procedure and HPLC conditions.

Our early trials with the μBondapak $C_{18}$ (Waters) column showed that although symmetrical peaks were obtained for CRA and TRA using the most commonly employed acetonitrile–aqueous ammonium acetate eluents, their resolution was not satisfactory. While the acetonitrile–aqueous acetic acid systems allowed good separation of CRA and TRA, both peaks showed tailing. Noticeable improvement in resolution and tailing was obtained using an acetonitrile–aqueous 0.5% acetic acid (60:40, v/v) eluent when modified with 0.05% (w/v) sodium hexanesulfonate. However, this system could not resolve TRA from retinol, which is endogenous in serum. Good baseline separation of TRA, retinol, CRA and the metabolite 4-oxo-13-*cis*-retinoic acid (OCRA) was obtained using Zorbax $C_{18}$ column (DuPont) and acetonitrile–aqueous 0.5% acetic acid (85:15, v/v) eluent containing 0.05% (w/v) sodium hexanesulfonate (Fig. 2). The major metabolite in human plasma after oral administration of CRA is OCRA [2,4]; TRA and its 4-oxo metabolite are present only at very low concentrations. Hence the above HPLC system performs well for the assay of CRA plasma and serum if the sample preparation adequately removes the matrix components.

*Sample and standard preparation*

The deproteination of plasma and serum by simple treatment with solvents (acetonitrile, methanol) with or without buffer modifiers have been used in the analysis of CRA and metabolites [7,12,21]. In our experience, the use of acetonitrile alone, while appearing satisfactory for deproteination, led to high variability in CRA assays of plasma. Improved precision in assay results as well as quick and very effective deproteination was achieved by a mixture of acetonitrile–perchloric acid–acetic acid. The addition of this mixture to both standards and samples appears to have stabilizing influence on CRA which improved both precision and linearity of response.

Full details of samples and standards preparation are given in the experimental section. Typical chromatograms of CRA spiked serum samples are shown in Fig. 3. A peak due to endogenous retinol was observed in all samples. CRA and internal

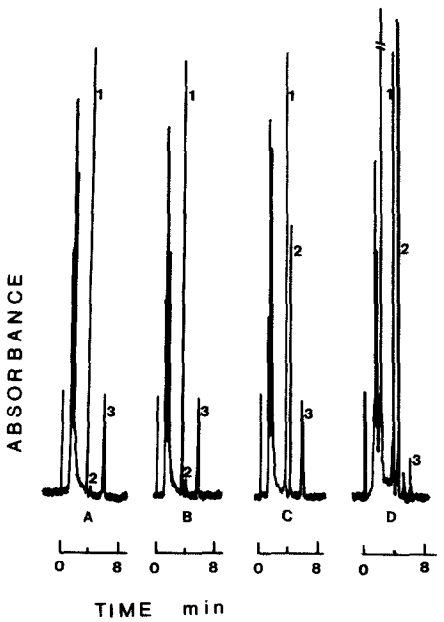


Fig. 3. Chromatograms of serum CRA standards (A, B, C) and clinical samples (D). CRA standards: A = 9.7 ng/ml; B = 19.4 ng/ml and C = 388 ng/ml. Clinical sample: plasma sample from volunteer treated with a single oral dose of 40 mg CRA. Detector wavelength 365 nm. See text for other HPLC conditions and the procedures for sample and standards preparation.

standard (9-methylanthracene) peaks in these chromatogram are well resolved from each other and from TRA, OCRA and retinol. CRA peak at the lowest CRA concentration, 9.7 ng/ml is easily noticeable.

A typical chromatogram of plasma from volunteer dosed orally with CRA is shown in Fig. 3D. The CRA and internal standard peaks are clearly separated from the metabolite and matrix component peaks. The CRA peak purity was further checked by monitoring the peak heights at two wavelengths, 365 nm and 313 nm. For six plasma samples from a volunteer (1.5 to 6 hr after dosing orally with 40 mg of CRA), the average peak height ratio was 2.46 [relative standard deviation (R.S.D.) 1.6%] compared to 2.43 (R.S.D. 1.6%) observed for three serum CRA standards.

*Linearity and limit of detection*

The calibration curve from spiked serum standards was linear over the range 9.7 to 2912 ng CRA/ml. Linear regression analysis of data on ten standards in this range, each analyzed in duplicate, gave a correlation coefficient 0.999, slope 0.0158 ng/ml and $y$-intercept 0.0069. Similar study with standards in a slightly narrower concentration range 50 to 2000 ng CRA/ml (6 standards, each analyzed in duplicate) led to a correlation coefficient greater than 0.999, on 7 different days performed over a period of 12 weeks.

Serum samples from ten separate individuals were spiked separately with CRS standard in methanol or with methanol only and analyzed without the use of internal standard in the HPLC method.

TABLE I

STABILITY OF CRA AT LOW CONCENTRATION IN SERUM AT −90°C

CRA concentration (theory) 157 ng/ml.

| Storage time (weeks) | Assay (ng/ml) | | | | | Mean | R.S.D. (%) | Initial (%) |
|---|---|---|---|---|---|---|---|---|
| | Individual results | | | | | | | |
| 0 | 163 | 162 | 160 | 158 | 161 | 160.8 | 1.2 | (100) |
| 4 | 181 | 158 | 173 | 165 | 165 | 168.4 | 5.2 | 104.7 |
| 7 | 157 | 164 | 158 | 161 | 153 | 158.6 | 2.6 | 98.6 |
| 14 | 159 | 159 | 164 | 156 | 161 | 159.8 | 1.8 | 99.4 |
| 28 | 162 | 160 | 159 | 163 | 163 | 161.4 | 1.1 | 100.4 |
| 43 | 169 | 167 | 163 | 165 | 164 | 165.6 | 1.5 | 103.0 |
| 56 | 161 | 160 | 158 | 164 | 162 | 161.0 | 1.4 | 100.1 |
| 84 | 155 | 160 | 157 | 156 | 152 | 156.0 | 1.9 | 97.0 |
| Inter-day R.S.D. (%) | 6.1 | 1.8 | 3.2 | 2.4 | 3.1 | | | |

TABLE II

STABILITY OF CRA AT HIGH CONCENTRATION IN SERUM AT −90°C

CRA concentration (theory) 1253 ng/ml.

| Storage time (weeks) | Storage | | | | | Mean | R.S.D. (%) | Initial (%) |
|---|---|---|---|---|---|---|---|---|
| | Individual results | | | | | | | |
| 0 | 1298 | 1244 | 1272 | 1253 | 1257 | 1265 | 0.2 | (100) |
| 4 | 1281 | 1243 | 1211 | 1304 | 1258 | 1259 | 2.8 | 99.5 |
| 7 | 1271 | 1238 | 1248 | 1226 | 1241 | 1245 | 1.3 | 98.4 |
| 14 | 1236 | 1263 | 1225 | 1252 | 1219 | 1240 | 1.5 | 98.0 |
| 28 | 1298 | 1271 | 1282 | 1352 | 1267 | 1294 | 2.7 | 102.3 |
| 43 | 1286 | 1320 | 1320 | 1311 | 1275 | 1302 | 1.6 | 102.9 |
| 56 | 1262 | 1268 | 1258 | 1256 | 1275 | 1264 | 0.4 | 99.9 |
| 84 | 1251 | 1241 | 1207 | 1208 | 1214 | 1224 | 1.7 | 96.8 |
| Inter-day R.S.D. (%) | 1.7 | 2.2 | 3.1 | 3.8 | 1.9 | | | |

The methanol spiked samples showed no detectable CRA peak while the samples spiked at a level 12.1 ng CRA/ml in serum gave an average peak height response of 29 with a standard deviation of 11.6 ($n$ = 10). These data suggest that CRA at 12 ng/ml level can be easily detected.

*Recovery and precision*

Two sets of CRA standards, one with and another without pooled serum, were prepared. Each set contained twelve standards, two at each concentration of 52.2, 104.4, 208.9, 417.7, 1044 and 2089 ng CRA/ml. All standards were treated the same prior to HPLC analysis, except that the standards without serum were not centrifuged since they require no clarification. Essentially identical linear response was observed with both sets. The CRA recovery of serum standards calculated using the linear regression equation of non-serum standards gave an average value of 100.5% ($n$ = 12, R.S.D. 1.9%) which demonstrates excellent recovery of CRA by this sample preparation method.

Two CRA spiked samples in pooled serum were prepared to contain 157 and 1253 ng CRA/ml and ten replicate analyses were performed on each sample using serum CRA standards. The results for the 157 ng/ml sample showed intra-day assay precision of 2.1% R.S.D. ($n$ = 10, recovery 101.6%), and 1.8% R.S.D. ($n$ = 10, recovery 100.2%) was found for the 1253 ng/ml sample. Additional discussion on intra-day precision and also inter-day precision data is presented in the next section.

*Stability of CRA in serum*

Serum CRA solutions (0.5 ml) at concentrations of 157 ng/ml and 1253 ng/ml were aliquotted into 4-ml vials, the head space in the vial was flushed with argon, and the vials were tightly capped and stored at −90°C until analysis. Five vials at selected storage times were analyzed independently. The data are summarized in Tables I and II and show that CRA is stable in serum for extended periods. Also, the data reveal that % R.S.D. ($n$ = 5) is in the range 0.2 to 5.2 for the intra-day analysis. The mean % R.S.D. for the 8 days is 2.1% at 157 ng/ml concentration and 1.5% at 1253 ng/ml concentration.

The excellent stability of CRA in serum at −90°C, as shown in Tables I and II allows us to use the same data for assessing the inter-day precision of the method. Calculating the % R.S.D. for assay results in each column (one result from each of 8 days) gave values of 6.1, 1.8, 3.2, 2.4 and 3.1 with a mean % R.S.D. of 3.3 for the 157 ng/ml samples. Similarly, the % R.S.D. values for the 1253 ng/ml samples are 1.7, 2.2, 3.1, 3.8 and 1.9, with a mean % R.S.D. of 2.5.

*Stability of processed samples*

The stability of deproteinated samples (in contact with the protein mass at the bottom of the vial) at

room temperature was studied by analyzing five processed plasma samples from a single subject, initially and 20 h later using freshly prepared serum CRA standards. These samples which had CRA concentrations in the range 694 to 2083 ng/ml were found to degrade in 20 h to 97.4% of initial ($n$ = 5, R.S.D. 1.3%).

*Advantages of the current method*

Simplicity in both the sample preparation and isocratic reversed-phase HPLC analysis are the major benefits of the method. The deproteination of plasma or serum samples is achieved efficiently by simple, addition of acetonitrile and perchloric acid (in acetic acid). The sample is handled in one 4-ml vial for the entire analysis, which made it very easy to minimize losses due to adsorption to surfaces and also protect CRA and metabolites from degradation by light and air. The excellent accuracy and precision achieved by this method is better than most published methods. The only methods which show comparable performance [14,18,19] are more complex, requiring sample preparation using solid phase or solvent extraction and chromatography employing gradient elution.

## CONCLUSION

The isocratic reversed-phase HPLC method described for the determination of CRA in serum is sensitive, precise and accurate. The simple sample preparation procedure coupled with the relatively fast isocratic HPLC analysis (10 min) makes it highly suited for monitoring serum CRA levels in clinical studies.

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