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Simple quantification of endogenous retinoids in bovine serum by high-performance liquid chromatography – diode-array detection

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Abstract

A high-performance liquid chromatographic method with diode-array detection (HPLC–DAD) was developed for the simultaneous quantification of endogenous retinoids in bovine serum. The described technique was initially based on an extraction procedure and reversed phase HPLC method designed for human serum, optimised and validated in the bovine. Following denaturation of 2 ml samples, a one-step liquid–liquid extraction was performed. Analytes were separated on a symmetry C_{18} column in a total run time of 60 min. The retinoids were monitored at an optimal wavelength of 350 nm as chosen from the retinoid spectra. Retinal was added as internal standard because of its intermediate polarity. Peak-height and peak-area ratios were linear over a concentration range from 1 to 10 ng/ml for 13-*cis*-, 9-*cis*-, and all-*trans*-retinoic acid, 100 to 600 ng/ml for retinol and 10 to 100 ng/ml for retinyl palmitate. Coefficients of variation were acceptable for both within-day and day-to-day assays. To allow the simultaneous analysis of the endogenous retinoids with a wide polarity range, recoveries were compromised. Nevertheless they systematically exceeded 60%. The proposed method is simple, specific and enables fast and accurate routine quantification of acid, alcoholic and esteric forms of retinoids. It was successfully applied to analyse 21 bovine serum samples from a clinical study. Simultaneous assay of endogenous retinoids has not yet been performed in cows.
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Keywords: Vitamin A; Retinoic acid isomers; Retinol; Retinyl palmitate; Bovine serum; HPLC–DAD

1. Introduction

Retinoids in the diet are taken up mainly as carotenoids (precursors of retinol), that are exclusively synthesised by photosynthetic microorganisms and by plants. The skeleton of natural retinoids is made up of a non-aromatic six-carbon ring structure with a polyprenoid side chain which is terminated with a carbon-oxygen functional group (Fig. 1). Following uptake, the cleavage of carotenoids to retinol or Vitamin A is the first step of a complex metabolic process. The storage forms of retinol in the liver and adipose tissue are the lipophilic retinyl esters, mainly consisting of retinyl palmitate. Other important metabolites of retinol are the oxidative intermediate retinal, the aldehyde form of retinol, and the further oxidation product retinoic acid, the carboxylic acid form of retinol [1]. Retinoic acid is biologically active at nanomolar concentrations in many tissues. Isomerisation of retinoic acid results in an array of

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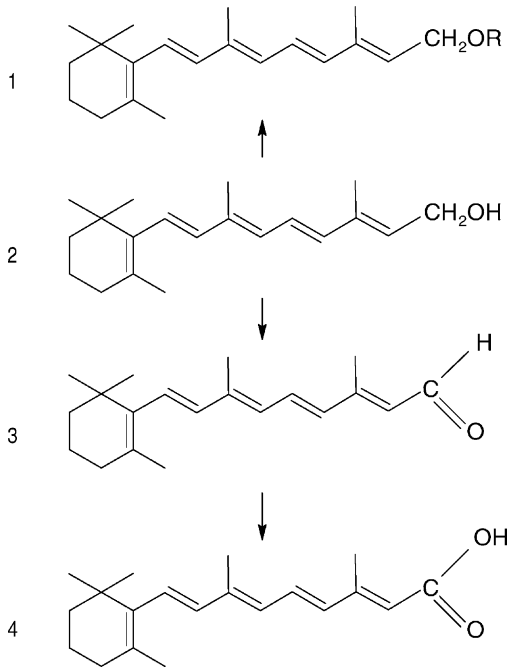

Fig. 1. Chemical structures of retinoids: (1) retinyl ester; (2) retinol; (3) retinal; (4) retinoic acid.

active acid metabolites such as 13-*cis*-, 9-*cis*-, and all-*trans*-retinoic acid. Natural retinoic acid isomers are characterised by varying effects due to their different mechanisms of receptor-mediated action.

In general, Vitamin A (retinol) and its acid metabolites (retinoic acids) are essential for growth and survival. Collectively referred to as retinoids, they are required for normal vision, differentiation and growth, and have recently been shown to modulate immune function and infectious disease susceptibility [2]. Documented effects of Vitamin A deficiency include impaired antibody production, decreased contact sensitivity to antigen and increased susceptibility to bacterial, viral, and parasitic infections. Repletion of Vitamin A in deficient animals partially or completely restores immune competency and disease resistance. In high yielding dairy cows remarkable changes in plasma Vitamin A occur during the periparturient period. The concentration of plasma retinol has been reported to decrease to 50% of the prepartal values immediately after parturition [3–5]. Interestingly, Horst et al. [6] described that the concentration of retinoic acid isomers increased dramatically during the same period, suggesting a shift in the metabolic pathway of retinoids.

To the best of our knowledge, no simultaneous quantification of endogenous retinoids in bovine serum or plasma has been described. The few research groups that studied endogenous retinoid levels in bovine species used separate HPLC methods to detect either retinol or retinoic acid isomers. Our aim was to develop a simple and sensitive method for the simultaneous determination of important natural retinoids in bovine serum. Although they share the same molecular skeleton, retinoids vary considerably in their physical and chemical properties. The polarity and thereby the solubility of various retinoids range from very soluble to insoluble in polar solvents like water and vice versa in apolar solvents like hexane [7]. Therefore, the development of a simultaneous determination method for retinoids always is an analytical challenge.

In contrast to the bovine, many HPLC methods for the quantification of retinoids in human serum or plasma have been reported [8]. However, most of these methods cover only a limited polarity range of retinoids. Protocols suitable for the analysis of polar retinoic acid metabolites cannot simultaneously detect the apolar retinyl esters [9–12], whereas methods that succeed to separate the less polar retinol from the retinyl esters, fail to separate the more polar retinoic acid metabolites [13]. Papers that describe the simultaneous assay of a wide polarity range in retinoids, without having to compromise on aspects such as analysis time or recovery are very scarce [14,15]. A reliable and sensitive method suitable for the simultaneous quantification of 13-*cis*-, 9-*cis*-, and all-*trans*-retinoic acid, all-*trans*-retinol, and retinyl palmitate in bovine serum in a reasonable run time is presented. The extraction protocol was based on Meyer et al. [9] and further adapted, whereas the HPLC conditions were optimised starting from those recently published by Barua and Olson [14].

2. Experimental

2.1. Reagents

2.1.1. Solvents and reagents

The extraction and mobile phase solvents acetonitrile, acetic acid, dichloromethane, *n*-hexane,

methanol, 2-propanol, and water were HPLC grade (LiChrosolv® quality). Ammonium acetate was of pro analysis quality. All reagents were obtained from Merck (Darmstadt, Germany).

2.1.2. Standards

All-*trans*-retinol, all-*trans*-retinal and retinyl palmitate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 13-*cis*-, 9-*cis*-, and all-*trans*-retinoic acid, all-*trans*-acitretin, isopropyl-acitretin and retinyl acetate were generously provided by Hoffmann-La Roche (Basel, Switzerland).

2.1.3. Serum samples

Blood samples were taken by venopuncture in the V. Jugularis Externa. Glass tubes were protected from light, and allowed to clot for 30 min at 37 °C. Serum was separated from cells by centrifugation at 1000 × *g* for 20 min and at 4 °C. The serum was transferred into amberised glass vials and stored at −20 °C until analysis.

2.1.4. Working conditions

As a precaution to avoid photoisomerisation, all sample manipulations were carried out in amberised tubes under dim yellow light.

2.2. Standard solutions

Each retinoid standard was dissolved in methanol to produce stock solutions of 100 ng/μl. Working solutions of 10, 1, and 0.1 ng/μl were prepared by serial dilutions of stock solutions with methanol. Following flushing with nitrogen, stock and working solutions were stored at −20 °C.

Spiked serum samples for validation were prepared using a frozen serum pool from 10 clinically healthy non-lactating cows.

2.3. Extraction procedure

Extraction of retinoids from bovine serum involved protein precipitation, followed by a one-step liquid–liquid extraction. The final protocol was optimised for bovine serum based on the extraction of retinoids described for human plasma by Meyer et al. [9]. In a first step, protein denaturation of 2-ml samples was performed with 2.1 ml of acetonitrile following acidification with 100 μl of 2N acetic acid. After 2 min of vortex mixing, 1.0 ml of water and 7.5 ml of an extraction mixture consisting of a *n*-hexane and 2-propanol (6.5:1.5, v/v) were added. The sample was extracted by vortex mixing for 2 min. After centrifugation (1000 × *g*, 10 min, 4 °C) the organic layer was removed and evaporated in a solvent evaporator at 15 °C (Speedvac SVC200; Savant, Holbrook, US) connected to a refrigerated vapour trap RVT400 (Savant). The residue was dissolved in 200 μl of mobile phase solvent A by vortex mixing. Following centrifugation at 300 × *g* for 3 min, the supernatant was transferred into an autosampler vial and 150 μl was injected.

2.4. Chromatographic system and procedure

2.4.1. Apparatus

The HPLC system consisted of an intelligent gradient pump L-6200A, an autosampler AS-2000A, a diode-array detector type L-4500, and an interface D-6000 all from Merck/Hitachi (Darmstadt, Germany). The 3.0-mm i.d. × 150 mm analytical column was filled with an dimethyloctadecylsilyl bonded amorphous silica stationary phase (Symmetry C_{18}, 5 μm particle size; Waters Inc., MA, USA).

2.4.2. Mobile phase and gradient program

The mobile phase and gradient conditions were based on the method of Barua and Olson [14], and further optimised for serum of bovine species. The solvent system consisted of methanol–water (3:1, v/v) containing 10 mM ammonium acetate and 0.025% acetic acid (solvent A) and of methanol–dichloromethane (4:1, v/v) (solvent B). A linear gradient from 100% solvent A to 100% solvent B was applied over a period of 30 min, followed by isocratic elution with solvent B for an additional 15 min (Table 1). Between each run the analytical column was equilibrated with

Table 1
Composition of mobile phase

Time (min)	Solvent A (%)	Solvent B (%)
0.0	100	0
16.0	47	53
30.0	0	100
45.0	0	100
45.1	100	0
60.0	100	0

100% solvent A for 15 min (total run time 60 min). The flow rate was set at 0.4 ml/min and the detection wavelength between 320 and 380 nm (from 18 to 40 min). Monitoring wavelength was set at 350 nm.

2.5. Biological samples

Three clinically healthy heifers in early lactation were infected intramammarily with *Escherichia coli* bacteria. Blood samples were taken at times 0, 3, 6, 9, 24, 48 and 72 h following infection. Two millilitre serum aliquots were spiked with the internal standard. Subsequent extraction and chromatographic analysis were performed as described earlier.

3. Results and discussion

3.1. Optimisation of the extraction protocol

3.1.1. Serum versus plasma

Because problems with unidentified interferences typical for bovine plasma were initially observed, serum was the preferred matrix for further experiments. The choice of serum over plasma is further encouraged by Aebischer et al. [16]. These authors stated that although no statistical differences exist for vitamins and carotenoids, plasma levels for all analytes were slightly but systematically lower than the corresponding serum levels.

3.1.2. Sample volume and pH

In analogy to its human counterpart, naturally occurring retinoic acid isomers are present at low nanomolar concentrations in bovine serum [7]. In order to determine trace amounts of analytes in complex matrices like serum, a major enrichment is required. However, liquid–liquid extraction of retinoic acid from biological fluids such as serum is hampered by the fact that retinoic acid, in contrast to most other retinoids that are water-insoluble, is rather water-soluble under physiological pH conditions [17]. Retinoic acid will therefore not be extracted optimally when water-immiscible solvents such as *n*-hexane are used without adaptation of the pH. The extraction efficiency of acid retinoids can indeed be significantly improved by prior acidification of the water-phase. Acetic acid or an acetate buffer and hydrochloric acid are the most commonly described acidifying agents in literature [9,11,12,14]. In our protocol a pH of 3 in the aqueous phase was obtained through acidification with acetic acid. A second possibility to improve the detection of trace amounts of analytes, is the use of large sample volumes. Because an increase in sample volume is always accompanied by an increase in interferences of the extract, a maximum sample volume of 2 ml was used in our final protocol.

3.1.3. Denaturation conditions

Due to their lipophilic character, retinoids exhibit strong protein-binding to specific or aspecific serum protein carriers like retinol-binding protein (retinol carrier) and albumin (retinoic acid carrier), respectively. Bound analytes were released by protein denaturation with acetonitrile in combination with acetic acid. Acetonitrile was preferred over other organic solvents such as the most commonly used alcohols (ethanol and methanol), because it resulted in better precipitation of the serum matrix than ethanol. The release of retinyl esters from lipoproteins was also improved. This observation is supported by literature describing the insolubility of most lipid classes in acetonitrile, the precipitation of lipids and their adherence to the glass [18]. Teerlink et al. [11] reported the critical influence of the acetonitrile content for optimal denaturation, and the beneficial effect of simultaneous acidification. In analogy to these authors, an optimum percentage of 50% acetonitrile was withheld for our protocol.

3.1.4. Liquid–liquid extraction

The classical way of extracting retinoids is by addition of a water-immiscible solvent after vigorous shaking, centrifugation and removal of the organic face [2]. A mixture of *n*-hexane and 2-propanol (6.5:1.5, v/v) was preferred for an optimal extraction of the retinoids in bovine serum. This in analogy with the extraction as performed by Meyer et al. [9] and Rissler et al. [19]. Although repetition of the extraction procedure may be performed, the described method resulted in satisfactory recoveries.

3.2. Choice of an adequate internal standard

We first evaluated the possibility of a validation without the use of an internal standard. Although exceptional, this option has indeed been previously

described by Teerlink et al. [11] for the simultaneous analysis of multiple retinoids in human serum. However, preliminary validation results showed that the use of an internal standard was required to compensate for losses and other sources of variation. An ideal internal standard should be absent from the original sample, chemically and physically similar to the analyte (i.e. be itself a retinoid), be well resolved from all other chromatographic peaks, and should possess similar characteristics for extraction, chromatography and detection as the analytes [2]. Four retinoids were evaluated as potential internal standards in the following chronological order: the synthetic acid retinoid all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nona tetraenoic acid (i.e. all-*trans*-acitretin or Ro 10-1670), its corresponding isopropylester (isopropyl-acitretin or Ro 11-0249), retinyl acetate and finally all-*trans*-retinal (the aldehyde analogue of retinol). In theory, the latter compound was not suited as an internal standard because it can be formed endogenously by metabolism of retinol. Nevertheless, the fact that we still evaluated it can be motivated as follows: the three other candidate internal standards were either susceptible to interference with an unidentified serum compound (Ro 10-1670) or did not compensate equally well for the diverse polarity range of analytes (Ro 11-0249), or coeluted with retinyl palmitate (retinyl acetate). Most importantly, in none of the chromatograms from blank bovine serum sample extracts a signal with the same retention time or spectrum as retinal was detected. This latter observation was confirmed by literature data stating that retinal occurs in significant quantities only in ocular tissue [20]. All criteria for an ideal internal standard were therefore met by retinal. Moreover, no artefactual oxidation of retinal to retinoic acid during sample preparation was observed upon addition of increasing amounts of retinal to a blank serum sample. The only remaining drawback on the use of retinal, was that it did not compensate equally well for retinyl palmitate as for the other analytes.

3.3. Optimisation of chromatographic conditions

3.3.1. Mobile phase pH and gradient program

As mentioned, the extraction of acid retinoids was significantly improved by acidification of the aqueous phase. The change in pH shifted the carboxylic acid functional group to its non-ionised form. In analogy, acidification of the mobile phase was performed during the subsequent chromatographic step. It allowed homogenous interaction of all non-ionised retinoic acid molecules with the reversed phase stationary phase. Only a very small percentage of acid was added because an acid mobile phase pH degraded the acid-sensitive silica phase. However, this minimal amount of acetic acid in combination with a low molarity of ammonium acetate avoided tailing of the acid retinoids and masked the presence of residual silanol functions [14].

The multi-step gradient program was optimised to allow optimal resolution of all analytes in an acceptable run time of about 30 min. Additional time with 100% of solvent B was necessary to elute potentially interfering lipophilic compounds from the column, while the last 15 min with 100% A were required to re-equilibrate the system.

3.3.2. Selection of monitoring wavelength

Because of their conjugated polyene chains, retinoids absorb light maximally at high-ultraviolet (UV) wavelengths ranging from 325 to 370 nm. This wavelength range is specific as few other natural compounds absorb light appreciably within this range [18]. The choice of a photodiode-array detector provided the possibility of both quantitative detection and qualitative identification, comparing the spectra of chromatographic peaks to those of reference retinoid standard spectra. The detection of the different retinoid analogues required a compromise for the monitoring wavelength. From the individual standard retinoid spectra it was determined that a maximal sensitivity of the trace amounts of retinoic acids was obtained at 350 nm. In comparison, the same wavelength was used by Meyer et al. [9] and Miyagi et al. [12], while Teerlink et al. [11] and Barua and Olson [14] monitor retinoids in human plasma at 340 nm. A representative chromatogram obtained from a serum extract analysed using the optimised chromatographic conditions is shown in Fig. 2.

3.4. Method validation

3.4.1. Retention time

The variability in retention time was found to be minimal both within-day and between-day, with

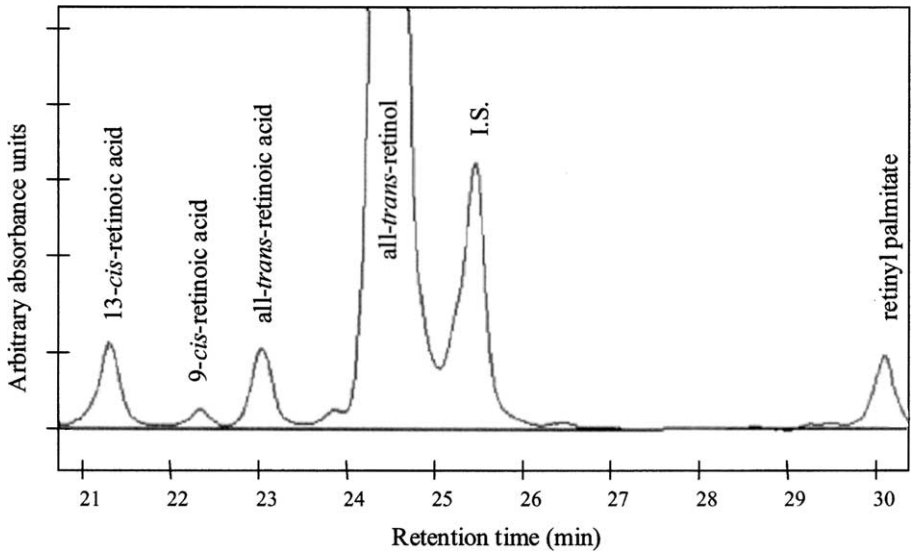

Fig. 2. Chromatogram of bovine serum spiked with retinoids (I.S.: internal standard).

coefficients of variation for all retinoids lower than 1 and 2%, respectively (Table 2).

3.4.2. Linearity range and calibration curves

Linearity was calculated by linear least-squares regression analysis plotting peak-height or peak-area analyte to internal standard ratios against the concentration of each analyte. Serum was spiked with 0, 1, 2.5, 5, 7.5, and 10 ng/ml for the three retinoic acid isomers; 0, 100, 150, 300, 450, and 600 ng/ml for retinol; 0, 10, 25, 50, 75, and 100 ng/ml for retinyl palmitate and 50 ng/ml for the internal standard. Additional analyses of linearity were performed on 10 consecutive days spiking serum from the same pool with 0, 1, 5 and 10 ng/ml for the three retinoic acid isomers; 0, 100, 300 and 600 ng/ml for retinol; 0, 10, 50, and 100 ng/ml for retinyl palmitate and 50 ng/ml for the internal standard. In all cases, with the exception of retinyl palmitate, an acceptable correlation coefficient was obtained. These *r*-values on average exceeded 0.999 for 13-*cis*- ($n = 10$); 0.994 for 9-*cis*- ($n = 6$), 0.999 for all-*trans*-retinoic acid ($n = 11$); and 0.992 for retinol ($n = 9$). An *r*-value of 0.961 was obtained for retinyl palmitate ($n = 9$).

Table 2
Reproducibility of retention times

Retinoid	Mean ± S.D. (min)	c.v. (%)
13-*cis*-retinoic acid	21.6 ± 0.21	0.95
9-*cis*-retinoic acid	22.7 ± 0.21	0.90
All-*trans*-retinoic acid	23.3 ± 0.20	0.86
Retinol	24.7 ± 0.15	0.59
Internal standard	25.7 ± 0.16	0.60
Retinyl palmitate	30.1 ± 0.14	0.46

Determined on a given day, $n = 10$.

3.4.3. Selectivity and specificity

No interfering peaks with a similar retention time but a different UV-spectrum as the analytes were observed in the chromatograms of the extracted serum samples. This indicates that the extraction and the chromatographic conditions ensured persistently clean chromatograms. More specifically, the use of an extra 15 min run time in the gradient program successfully prevented the occurrence of ghost peaks. In addition, no interfering peaks were observed in any of the clinical samples analysed as a preliminary application of the newly developed method.

3.4.4. Precision

To test for reproducibility, a pool of bovine serum was divided in three pools and spiked with each of the retinoid standards at three different concentration levels (low, medium, high). The added concentrations

Table 3
Within-day and day-to-day reproducibility, expressed as coefficients of variation values (%)

Retinoid	Low	Medium	High
Within-day			
13-*cis*-retinoic acid	8.33	7.98	4.69
9-*cis*-retinoic acid	4.49	8.23	5.84
All-*trans*-retinoic acid	11.05	7.87	6.08
Retinol	6.28	5.72	5.19
Retinyl palmitate	20.73	17.08	15.45
Day-to-day			
13-*cis*-retinoic acid	10.37	15.82	9.41
9-*cis*-retinoic acid	13.19	16.39	11.10
All-*trans*-retinoic acid	11.56	14.03	10.03
Retinol	13.53	14.22	10.77
Retinyl palmitate	21.97	13.53	13.99

$n = 10$ for all retinoids, except for retinyl palmitate $n = 8$.

for the low, medium and high concentration level were 1, 5 and 10 ng/ml for the retinoic acid isomers; 100, 300 and 600 ng/ml for retinol; and 10, 50 and 100 ng/ml for retinyl palmitate, respectively. The amount of internal standard added was kept constant for all samples. For the within-day reproducibility 10 samples of each concentration level were analysed on 1 day, while the day-to-day reproducibility was tested for each of the three concentration levels on 10 different days covering a 2-month period. The coefficients of variation (c.v. values), as determined from the height ratios, were satisfactory for all retinoids, even for the retinoic acid isomers which were present at trace amount levels only (Table 3). In general, the c.v. values were lower than 10% for the within-day, and lower than 15% for the day-to-day measurements. An exception was the c.v. value at the low concentration level of retinyl palmitate, which exceeded 15% both within-day and day-to-day.

3.4.5. Limit of quantification

The quantification limit of each retinoid was determined at a signal-to-noise ratio of 5/1. Following quantification limits were obtained: 1 ng/ml for 13-*cis*-, 3 ng/ml for 9-*cis*-, 1 ng/ml for all-*trans*-retinoic acid, 86 ng/ml for retinol and 17 ng/ml for retinyl palmitate, respectively. These data indicate that the analysis method allows the quantification of endogenous retinoid levels. The reference values found in the literature for the bovine range from 1 to 5 ng/ml for retinoic acid and from 150 to 600 ng/ml for retinol [4,6]. No values are available for retinyl palmitate in bovine serum.

3.4.6. Recovery

The recovery of each retinoid was determined at three concentration levels (low, medium, and high) and for at least seven samples. The signal of serum extracts to which increasing amounts of each retinoid were added, was compared to the signal of the corresponding standards injected directly into the HPLC system. Endogenous retinoid levels from blank serum were subtracted. The obtained recoveries are a compromise. Recovery for the acid isomers were on average 75, 73 and 64% for 13-*cis*-, 9-*cis*- and all-*trans*-retinoic acid, respectively, 57% for retinol and 61% for retinyl palmitate. Other authors report higher values but did not cover the wide polarity range of retinoids reported here, with the exception of Barua and Olson [14].

3.5. Analysis of clinical samples

Serum samples ($n = 21$) from a preliminary clinical study were analysed to evaluate the newly developed method. The obtained preliminary data indicate that endogenous pre-infection levels of retinoic acids and retinol are in good accordance with previously published data [5–7] for separate endogenous retinoids in the bovine. Although too few samples were included to allow for statistical interpretation of the results, our preliminary data indicate that serum retinol levels decrease early post-infection. Remarkably, there is no general hyporetinemia as the serum levels of the retinoic acid isomers and of retinyl palmitate are not concomitantly decreased. To the best of our knowledge this is the first report on endogenous retinoid levels during infection in the bovine.

4. Conclusion

Our aim was to develop a simple and sensitive identification and quantification method for the single-run analysis of the principal endogenous retinoids i.e. retinol, its three retinoic acid metabolites (13-*cis*-, 9-*cis*-, all-*trans*-retinoic acid), and its predominant retinylester metabolite (retinyl palmitate) in bovine serum. The HPLC–DAD procedure described in this paper provides a new useful tool for routine

monitoring of changes in endogenous retinoid levels, e.g. in bovine serum samples from infection studies. A few more efficient methods have been previously described for the analysis of individual retinoids or a single class of retinoids in the bovine (retinoic acid by Horst et al. [7]; retinol and β-carotene by Johnston and Chew [3]). Nevertheless, this is to the best of our knowledge the first report on the fast and accurate screening of a wide polarity range of endogenous retinoids in bovine serum. This method will be applied in a clinical study on the changes in pre- and post-infection levels of endogenous retinoids in the bovine during the periparturient period.

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