ELSEVIER

Journal of Chromatography A, 794 (1998) 245–251

JOURNAL OF CHROMATOGRAPHY A

Development of a method for quantitation of retinol and retinyl palmitate in human serum using high-performance liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry

Richard B. van Breemen[a,*], Dejan Nikolic[a], Xiaoying Xu[a], Yansan Xiong[a], Machteld van Lieshout[b], Clive E. West[b], Alexander B. Schilling[c]

[a]*Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 South Wood Street, Chicago, IL 60612-7231, USA*

[b]*Department of Human Nutrition, Wageningen Agricultural University, P.O. Box 8129, 6700 EV Wageningen, Netherlands*

[c]*The Hewlett-Packard Co., 1200 E. Diehl Road, Naperville, IL 60566, USA*

Abstract

A method for the quantitative analysis of the vitamin A compounds *all-trans*-retinol and *all-trans*-retinyl palmitate was developed using high-performance liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (APCI–LC–MS). Unlike previous quantitative mass spectrometric methods for vitamin A, HPLC separations were carried out using a C_{30} reversed-phase column instead of GC separation. Because no sample hydrolysis or derivatization was necessary, retinyl palmitate was preserved for analysis instead of being hydrolyzed to retinol. Human serum was analyzed following simple hexane extraction without saponification or any additional purification. A comparison of APCI and electrospray ionization showed that only APCI produced a linear response over all four orders of magnitude of retinol and three orders of magnitude of retinyl palmitate concentrations. Selected ion monitoring of the fragment ion of m/z 269 was used for APCI quantitation of both retinol and retinyl palmitate, since it was the base peak and the only abundant ion in the mass spectra of both compounds and the internal standard, retinyl acetate. The ion of m/z 269 corresponded to loss of water, loss of palmitic acid, or elimination of acetic acid from the protonated molecules of retinol, retinyl palmitate and retinyl acetate, respectively. The limit of detection of APCI–LC–MS for *all-trans*-retinol and *all-trans*-retinyl palmitate was determined to be approximately 34 fmol/μl and 36 fmol/μl (0.670 pmol *all-trans*-retinol and 0.720 pmol *all-trans*-retinyl palmitate injected in 20 μl on-column), respectively. The limit of quantitation was approximately 500 fmol/μl and 250 fmol/μl (10 pmol and 5 pmol injected in 20 μl on-column) for retinol and retinyl palmitate, respectively.

Keywords: Atmospheric pressure chemical ionization; Electrospray ionization; Mass spectrometry; Retinol; Retinyl palmitate; Vitamin A

1. Introduction

Retinoids include vitamin A compounds such as retinol and retinal, their derivatives and analogs and related molecules that bind to retinoid receptors and elicit specific biological responses [1]. Many natural and synthetic retinoids have been shown to exert beneficial effects on reproduction, vision, skin and

*Corresponding author.

0021-9673/98/$19.00
PII S0021-9673(97)01138-2

immune function, and inhibit cell proliferation and differentiation and possibly prevent cancer [1,2]. However, overdoses of vitamin A retinoids can cause hypervitaminosis and associated toxicities such as teratogenesis. Because retinoids are important dietary as well as therapeutic compounds, their identification and quantification in foods and biological tissues is critically important. Reversed- or normal-phase high-performance liquid chromatography (HPLC) is the preferred separation and purification technique for retinoids [3,4], because the mild conditions of HPLC are compatible with the heat, light and oxygen sensitive properties of retinoids.

The conjugated polyene structure of retinoids results in strong molar absorptivities at UV and often visible wavelengths, which have been utilized in sensitive HPLC spectrophotometric UV–Vis assays for the detection and quantification of retinoids [3,4]. Similarly, UV–Vis photodiode array detection has been used to assist in retinoid identification. However, retinoid separation and identification may be complicated by the occurrence of *cis* and *trans* isomers, which is another consequence of the polyene structures of these compounds, and by the formation of retinyl esters. Therefore, a complementary identification technique such as mass spectrometry should be utilized during HPLC to confidently identify retinoids. However, quantitative analyses of retinoids using mass spectrometry (MS) typically utilize gas chromatography–mass spectrometry (GC–MS), which requires hydrolysis of retinyl esters and derivatization of retinol and retinoic acid [5,6]. The hydrolysis step eliminates all information regarding retinyl esters, and the high temperature of the GC oven destroys all information concerning *cis* and *trans* geometric isomers.

Retinoic acid and its metabolites, often as their pentafluorobenzyl ester derivatives, have been analyzed by using various liquid chromatography–mass spectrometry (LC–MS) methods including direct liquid introduction [7,8], thermospray [9] and continuous-flow fast atom bombardment [10]. In 1996, we reported for the first time that electrospray LC–MS may be used for the analysis of a variety of underivatized retinoids including retinoic acid, retinol, retinal and retinyl acetate [11]. In the current investigation, we explore the application of another atmospheric pressure ionization technique, atmospheric pressure chemical ionization (APCI), to the LC–MS analysis of underivatized retinoids extracted from human serum. Furthermore, we report a method for APCI–LC–MS quantitative analysis of retinol and retinyl palmitate that utilizes a simple hexane extraction of serum followed by on-line C_{30}-reversed-phase HPLC separation with APCI mass spectrometric detection.

2. Experimental

APCI mass spectra were acquired using a Hewlett-Packard (Palo Alto, CA, USA) G1946A LCMSD quadrupole mass spectrometer equipped with a Series 1100 HPLC system consisting of a binary pump, automatic solvent degasser and autosampler. Operating parameters of the APCI–LC–MS interface were optimized using flow injection of retinol in a mobile phase containing 50% solvent B (see HPLC conditions below) at 250 μl/min. Optimum conditions included a nitrogen nebulizer pressure of 30 p.s.i., vaporizer temperature of 200°C, corona current of 5.0 μA, nitrogen drying gas temperature of 250°C at a flow-rate of 7.0 l/min, capillary (VCAP) voltage of 3800 V and a fragmentor voltage (used for in-source collision induced dissociation) of 60 V (p.s.i.=6894.76 Pa). Mass spectra were acquired over the scan range m/z 100–500 in approximately 1 s. Quantitative analysis was carried out using selected ion monitoring (SIM) of the abundant fragment ion of retinol, retinyl acetate and retinyl palmitate at m/z 269 with a dwell time of 200 ms. Electrospray mass spectra of retinol were obtained using flow injection on a Hewlett-Packard 5989B quadrupole mass spectrometer as previously described [11]. During preliminary studies, APCI mass spectra were obtained using a Hewlett-Packard 5989B equipped with an Analytica (Branford, CT, USA) APCI ion source. Since the limit of detection of the G1946A LCMSD was found to be approximately 80-fold lower (more sensitive) and the baseline more stable than the 5989B, the G1946A LCMSD mass spectrometer was used for all quantitative analyses using APCI.

HPLC separations were carried out using a C_{30} reversed-phase column, 10 cm×2.0 mm (YMC, Wilmington, NC, USA), with a solvent system

consisting of a linear gradient from either 30–90% solvent B in 30 min or 20–90% solvent B in 25 min. Solvent A contained methanol–water–acetic acid (50:50:0.5, v/v/v), and solvent B consisted of methanol–methyl-*tert*.-butyl ether–acetic acid (50:50:0.5, v/v/v). Acetic acid functioned both as an ion-pair reagent during reversed-phase HPLC and facilitated formation of protonated retinoids, $[M+H]^+$, during positive ion APCI. Both gradients produced good separations and chromatograms from each are presented in Section 3. Two different gradients were used during this study because these separation methods were developed in different laboratories using two different mass spectrometers. The benefit of this approach is that the methods and instrumentation may be compared within a single study. Although the two gradients were equally effective, the two mass spectrometers were not equally sensitive as discussed above.

HPLC solvents were purchased from Fisher Scientific (Springfield, NJ, USA), retinoids were purchased from Sigma (St. Louis, MO, USA), and $^{13}C_6$-retinyl palmitate was a gift from Prof. J. Lugtenburg, Leiden University (Leiden, The Netherlands). Retinoid standard solutions were prepared in solvent B and contained retinol, retinyl palmitate and 1.84 pmol/μl retinyl acetate as internal standard. Serum (1 ml), containing retinyl acetate as internal standard (1.84 pmol/μl), was diluted with saline (1 ml, NaCl 9 g/l) and proteins were precipitated with ethanol (1 ml). The solution was extracted three times with hexane (2 ml). The combined hexane fractions were evaporated to dryness in a centrifuge/vacuum concentrator, and then redissolved in 100 μl of solvent B. For each LC–MS analysis, 20 μl of a standard mixture or serum extract was injected onto the HPLC column. Although this injected quantity represents only 200 μl of the original serum, 1 ml of serum was extracted so that multiple injections could be made from each sample for greater confidence. If desired, a smaller volume of serum could be extracted. The peak areas of retinol and retinyl palmitate in the mass chromatograms were calculated using Hewlett-Packard Chemstation software and then normalized to the area of the internal standard peak, retinyl palmitate, which was present at a constant concentration (1.84 pmol/μl) in all samples and standards. Calibration curves were generated using linear regression analysis with all values weighted equally.

Retinoid standard solutions were prepared in solvent B instead of human serum, because serum contains substantial quantities of retinol and highly variable amounts of retinyl palmitate. In order to verify that no components of serum would interfere with retinoid quantitation using LC–MS, serum samples and solvent blanks were spiked with $^{13}C_6$-retinyl palmitate at two different concentrations, 4 and 20 pmol/μl, and the usual amount of retinyl acetate. After extraction, both samples were analyzed using LC–MS with SIM at m/z 275 for the labeled compound and m/z 269 for the unlabeled internal standard.

The extraction efficiency was determined by measuring the amounts of retinol and retinyl palmitate in a serum extract, then spiking aliquots of the serum with different amounts of retinol and retinyl palmitate, extracting and determining the amounts of retinol and retinyl palmitate in the spiked samples based on a standard curve. The standard curve was prepared using retinoids dissolved in mobile phase B. Extraction efficiencies ranged from 95% for retinyl acetate to 101% for retinol.

3. Results and discussion

APCI was selected for the development of a quantitative LC–MS method because of its wide dynamic range and linearity of detector response. Although electrospray is a sensitive alternate ionization method for LC–MS of retinoids [11], it lacks linearity of detector response over a wide range of retinoid concentrations. For example, Fig. 1 shows a standard curve for the electrospray mass spectrometric analysis of retinol over the concentration range 1.0–2000 pmol/μl. The base peak at m/z 269 in the positive-ion electrospray mass spectrum of retinol (corresponding to loss of water from the protonated molecule) was recorded using SIM. The slope of the electrospray standard curve for retinol was greatest at low concentration, decreased rapidly up to approximately 80 pmol/μl, and then continued to decrease (although less rapidly) up to 2000 pmol/μl. In contrast, the APCI standard curves in Fig. 2 showed excellent linearity with a correlation coefficient, r^2,

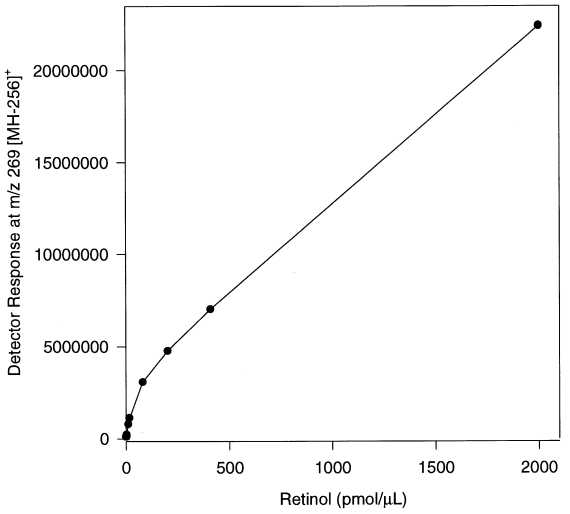

Fig. 1. Standard curve for analysis of *all-trans*-retinol using positive-ion electrospray mass spectrometry with flow injection. The base peak of the mass spectrum at m/z 269, $[MH-18]^+$, was recorded using SIM. Note that the electrospray curve shows a non-linear response.

of better than 0.999 over the concentration range 0.0524–3400 pmol/μl for *all-trans*-retinol and 0.0249–28.3 pmol/μl for *all-trans*-retinyl palmitate.

Examples of APCI–LC–MS analysis of standard mixtures of *all-trans*-retinol, *all-trans*-retinyl acetate and *all-trans*-retinyl palmitate are shown in Fig. 3. Retinyl acetate was added to the samples as an internal standard. Each compound is well resolved from the others in the chromatograms and shows a single peak for the *all-trans* isomer without evidence of *cis* forms. If samples are exposed to light or air (such as sitting in an autosampler for several hours), then *cis* isomers would be formed and would be evident in these standard chromatograms. The mass chromatogram in Fig. 3B shows amounts of retinol and retinyl palmitate near the limit of detection (defined as a signal-to-noise of at least 5:1) corresponding to 0.672 pmol and 0.719 pmol, respectively, injected on the column.

Because all human serum samples contain high concentrations of retinol and variable amounts of retinyl palmitate (highest after meals), no blank serum was available for preparation of standards or controls. For example, the LC–MS analysis of a human serum extract in Fig. 4A shows a large peak

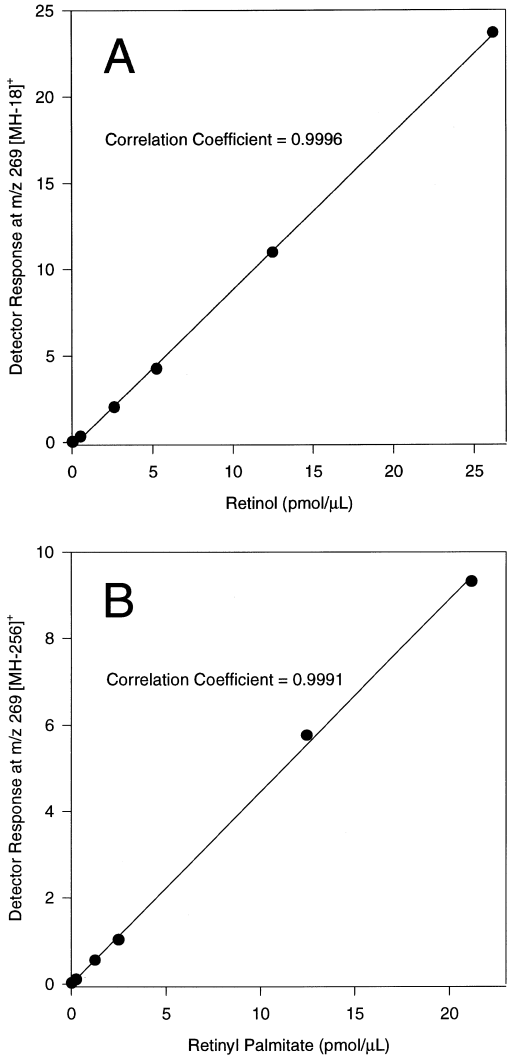

Fig. 2. Standard curves for the analysis of (A) *all-trans*-retinol and (B) *all-trans*-retinyl palmitate using positive-ion APCI–LC–MS. The base peak in each mass spectrum at m/z 269 was recorded using SIM. Note that each retinoid gave a linear mass spectrometer response over the entire range of concentrations investigated.

for retinol as well as a peak corresponding to retinyl palmitate. Therefore, all standards were prepared in mobile phase. To investigate whether any signals at m/z 269 could be detected in the solvent used for the preparation of standards, a solvent blank was analyzed by LC–MS, and the resulting chromatogram is shown in Fig. 4B. No peaks at m/z 269 were observed in the blank. As an additional control

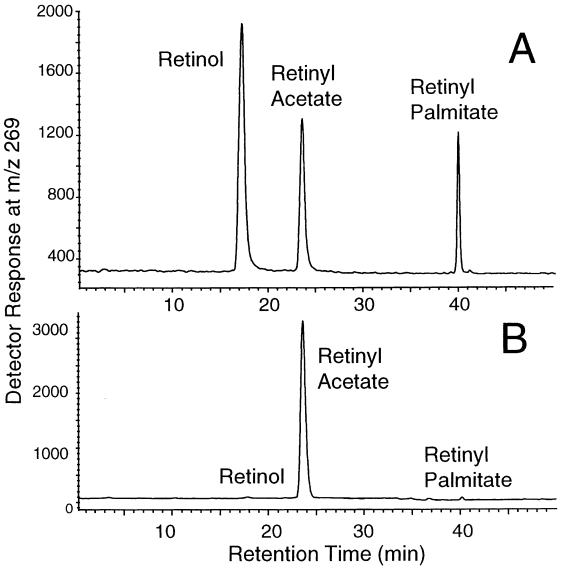

Fig. 3. Mass chromatograms for the positive ion APCI–LC–MS analysis of (A) a standard mixture containing 52.4 pmol *all-trans*-retinol, 36.8 pmol *all-trans*-retinyl acetate and 24.9 pmol *all-trans*-retinyl palmitate in 20 μl; and (B) retinol and retinyl palmitate at the limit of detection, 0.672 pmol and 0.719 pmol, respectively, and 36.8 pmol retinyl acetate as an internal standard. A C_{30} reversed-phase column was used for retinoid separation with a gradient from 30–90% solvent B in 30 min, and a Hewlett-Packard G1946A LCMSD mass spectrometer was used for detection.

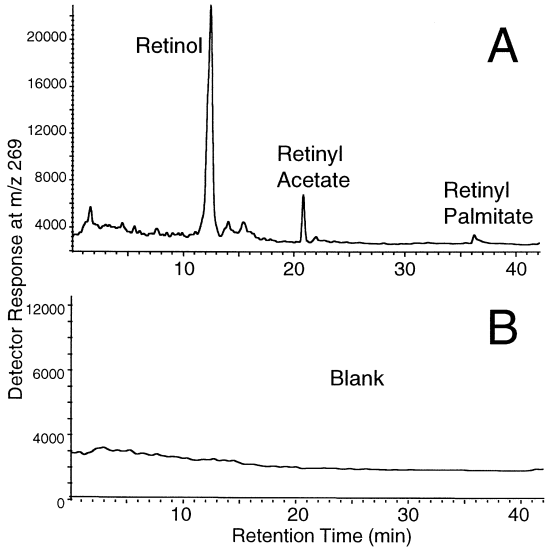

Fig. 4. Positive ion APCI LC–MS analyses of (A) an extract of 200 μl of human serum recorded using scanning mode. The computer-reconstructed mass chromatogram at *m/z* 269 is shown; and (B) a solvent blank showing no signals at *m/z* 269. The HPLC gradient was from 20–90% solvent B in 25 min, and a Hewlett-Packard 5989B mass spectrometer was used for detection.

experiment, $^{13}C_6$-retinyl palmitate at two different concentrations and unlabeled retinyl acetate as an internal standard were added to serum or solvent B, and the samples were extracted and then analyzed by using LC–MS with selected ion monitoring at *m/z* 275 for the $^{13}C_6$-labeled compound. The values of labeled retinyl palmitate in the solvent and in the serum were measured to be within 8% (20 pmol/μl level) and 13% (4 pmol/μl level) of each other ($n=2$), with the serum values being slightly higher than the solvent samples. Because these differences are at or within the error of the method (standard deviation for these measurements was ±12%), the use of mobile phase instead of serum for the preparation of standards was found to be acceptable. As a final control, serum was extracted and analyzed without the addition of the retinyl acetate internal standard, and as expected, no peak was observed between 20 and 25 min corresponding to retinyl acetate.

SIM of the fragment ion of *m/z* 269 was used for the quantitative analysis of both retinol and retinyl palmitate, because it was the base peak of the positive ion APCI mass spectra of both compounds. In addition, the fragment ion of *m/z* 269 was the base peak of the APCI mass spectrum of the internal standard, retinyl acetate. Positive-ion APCI mass spectra of all three compounds are shown in Fig. 5. The ion of *m/z* 269 was formed in the APCI ion source by elimination of water, acetic acid or palmitic acid from the protonated molecules of retinol, retinyl acetate or retinyl palmitate, respectively. These APCI mass spectra show higher abundances of the *m/z* 269 ion and less abundant intact protonated molecules than corresponding positive ion electrospray mass spectra of retinol and retinyl esters [11].

Additional LC–MS analyses of hexane extracts of human serum (equivalent to 200 μl serum injected on-column) are shown in Fig. 6. The concentration of retinol in each serum sample was determined to be 1.56 and 1.04 μmol/l for Figs. 3A and 3B, respectively, which is within the expected normal adult values of 1.0 to 2.3 μmol/l [12]. In these samples,

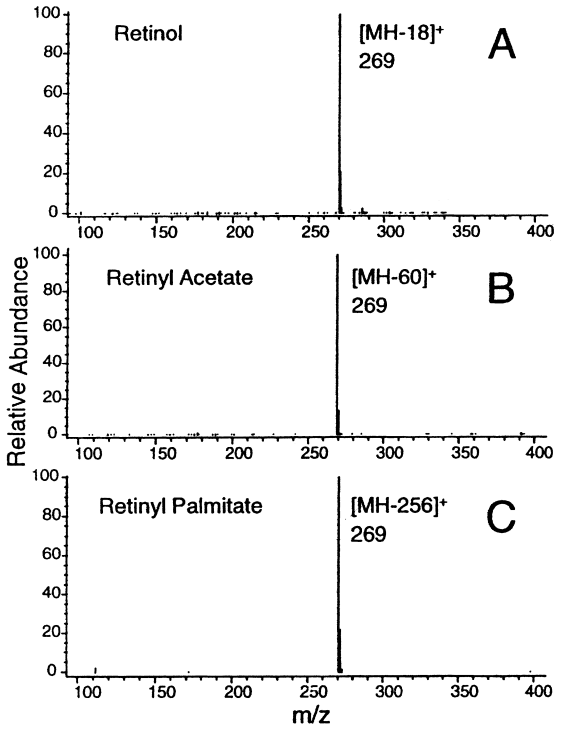

Fig. 5. Positive-ion APCI mass spectra recorded during the LC–MS analysis shown in Fig. 4A including (A) *all-trans*-retinol at 16.1 min, (B) *all-trans*-retinyl acetate at 21.9 min and (C) *all-trans*-retinyl palmitate at a retention time of 38.5 min. Note that all three retinoids fragmented during APCI to form a common base peak of *m*/*z* 269, which was used for SIM during quantitative analysis.

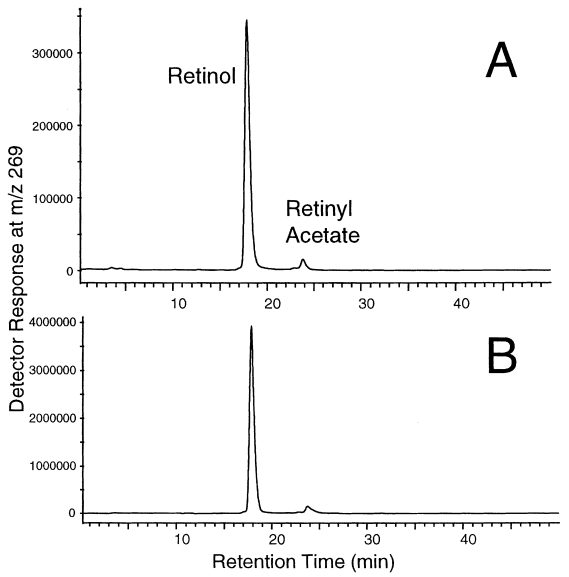

Fig. 6. Positive ion APCI LC–MS analyses of extracts of two different human serum samples (the amount injected onto the LC–MS for each analysis was equivalent to 200 μl of serum). The retinol peaks were calculated to correspond to serum retinol concentrations of (A) 1.56 μmol/l and (B) 1.04 μmol/l.

which were obtained from fasting adults, no retinyl palmitate was detected. During LC–MS, no co-eluting serum compounds were observed to interfere with the mass spectrometric detection. However, carbon deposits were observed to accumulate on the needle electrode of the APCI ion source, which gradually reduced sensitivity. The use of an internal standard helped to correct for changes in mass spectrometer response during the analysis of a set of samples. By wiping these deposits off of the needle after every 6–8 h of serum analysis, sensitivity could be maintained. No significant deposits were observed following analysis of standard samples. The ability to use the same C_{30} reversed phase column and solvents for both retinoid analysis and carotenoid LC–MS analysis [13,14] is of considerable economy and convenience. Although a microbore HPLC column would permit even less sample to be injected per analysis, a 2.0 mm I.D. C_{30} column with 20 μl injection volumes provided robust performance without clogging.

The limit of detection of APCI–LC–MS for *all-trans*-retinol and *all-trans*-retinyl palmitate (defined as a signal-to-noise of at least 5:1) was determined to be approximately 34 fmol/μl and 36 fmol/μl (0.670 pmol *all-trans*-retinol and 0.720 pmol injected in 20 μl on-column) for *all trans* retinol and *all trans* retinyl palmitate, respectively. Defining the limit of quantitation as a signal-to-noise ratio of 10:1, this value was determined to be less than or equal to 524 fmol/μl and 249 fmol/μl (10.5 pmol and 4.98 pmol injected in 20 μl on-column) for retinol and retinyl palmitate, respectively. The reproducibility of this quantitative LC–MS method was evaluated by comparing replicate LC–MS analyses for sample concentrations near the limit of quantitation and at concentrations tenfold higher. The coefficients of variation ($n=4$) for quantitation of 524 fmol/μl retinol and 249 fmol/μl retinyl palmitate were determined to be ±1.23% and ±12.5%, respectively. At tenfold higher concentrations, the coefficients of

variation were ±5.78% and ±6.73% ($n=4$) for retinol and retinyl palmitate.

4. Conclusions

APCI–LC–MS of retinoids provides a sensitive quantitative method that may be automated by use of autoinjection and autosampling. Serum preparation requires only simple hexane extraction. Since no saponification or sample derivatization is required, important dietary information such as the amount of retinyl palmitate (a retinol precursor that appears in the blood soon after a meal) may be obtained in addition to retinol concentration. Other mass spectrometric methods for retinol typically require sample derivatization for GC–MS [5,6] and often much more elaborate purification prior to GC–MS such as a preliminary HPLC purification step [6]. Alternatively, HPLC separation of retinoids has been used followed by elaborate sample processing prior to isotope ratio mass spectrometry [15]. Because retinoids are light, heat and air sensitive, a minimum of sample handling is preferred to insure sample stability and prevent the formation of artifacts.

This new APCI–LC–MS method provides a linear mass spectrometer response over a wide range of concentrations. This dynamic range combined with high sensitivity and mass spectrometric specificity suggest that APCI–LC–MS might be useful for studies in humans of vitamin A bioavailability using stable isotope-labeled retinoids. Similar mass spectrometric methods have been used for such studies [5,16,17], and experiments are in progress in our laboratories to explore the utility of APCI–LC–MS for investigations using stable isotope-labeled retinol and retinyl palmitate.

Acknowledgements

Use of the electrospray and APCI mass spectrometers was generously provided by the Hewlett-Packard Company.

References

[1] M.B. Sporn, A.B. Roberts and D.S. Goodman, The Retinoids: Biology, Chemistry and Medicine, Raven Press, New York, 2nd ed., 1994.
[2] J.A. Olson, in M.E. Shils, J.A. Olson and M. Shike (Editors), Modern Nutrition in Human Health and Disease, Lea and Febiger, Philadelphia, PA, 8th ed., 1994, p. 287.
[3] H.C. Furr, A.B. Barua and J.A. Olson, in M.B. Sporn, A.B. Roberts and D.S. Goodman (Editors), The Retinoids: Biology, Chemistry and Medicine, Raven Press, New York, 2nd ed., 1994, p. 179.
[4] R. Wyss, J. Chromatogr. B 671 (1995) 381–425.
[5] D.V. Reinersdorff, E. Bush, D.J. Liberato, J. Lipid Res. 37 (1996) 1875–1885.
[6] G.J. Handelman, M.J. Haskell, A.D. Jones, A.J. Clifford, Anal. Chem. 65 (1993) 2024–2028.
[7] B.E. Fayer, C.A. Huselton, W.A. Garland, D.J. Liberato, J. Chromatogr. 568 (1991) 135–144.
[8] U.B. Ranalder, B.B. Lausecker, C. Huselton, J. Chromatogr. 617 (1993) 129–135.
[9] S. Cotler, D. Chang, L. Henderson, W. Garland, C. Town, Xenobiotica 22 (1992) 1229–1237.
[10] K.L. Salyers, M.E. Cullum, M.H. Zile, Biochim. Biophys. Acta 1152 (1993) 328–334.
[11] R.B. van Breemen, C.-R. Huang, FASEB J. 10 (1996) 1098–1101.
[12] N.W. Tietz, Textbook on Clinical Chemistry, W.B. Saunders Co., New York, 1995.
[13] R.B. van Breemen, Anal. Chem. 67 (1995) 2004–2009.
[14] R.B. van Breemen, C.-R. Huang, Y. Tan, L.C. Sander, A.B. Schilling, J. Mass Spectrom. 31 (1996) 975–981.
[15] C.S. You, R.S. Parker, K.J. Goodman, J.E. Swanson, T.N. Corso, Am. J. Clin. Nutr. 64 (1996) 177–183.
[16] S.R. Dueker, A.D. Jones, G.M. Smith, A.J. Clifford, Anal. Chem. 66 (1994) 4177–4185.
[17] J.A. Novotny, S.R. Dueker, L.A. Zech, A.J. Clifford, J. Lipid Res. 36 (1995) 1825–1838.