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Determination of Triterpenic Acids in Human Serum by High-Performance Liquid Chromatography: Triterpenoid Interaction with Serum Protein

Mirela Rada,* Valentina Ruiz-Gutiérrez, and Ángeles Guinda

Food Characterization and Quality Department, Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), 4 Avenida Padre García Tejero, 41012 Sevilla, Spain

ABSTRACT: Terpenic acids are under development as therapeutic agents in numerous treatments. In support of pharmacokinetic and toxicological evaluations, a robust assay based on high-performance liquid chromatography (HPLC) was developed for the analysis of the terpenoids in human serum. For a clear understanding of the differences in biological activity of these compounds, the interactions between oleanolic or betulinic acids and human serum protein have been studied by ultraviolet−visible (UV−vis) absorption under physiological conditions. A combination of liquid/liquid extraction, centrifugation, and consecutive HPLC resulted in simultaneous separation, identification, and quantification of the oleanolic, betulinic, and ursolic acids. The validity of the developed method was established by determining linearity, recovery, precision, accuracy, limit of detection, and quantification. Detection limits were in the range of 3.3−4.3 ng/mL, and linearity values ranged up to 1 μg/mL. The repeatability of the method was good. All compounds can be well-distinguished by order of elution during liquid chromatography. The pentacyclic triterpenoids have been identified by retention time comparison to pure standards and quantified by an internal standard. The results by UV−vis absorption spectra experiments (240−340 nm) indicate that protein structures have been perturbed in the presence of oleanolic and betulinic acids.

KEYWORDS: Terpenic acids, HPLC, terpenoid, human serum

INTRODUCTION

Oleanolic acid (OA, 3β-hydroxyolean-12-en-28-oic acid), betulinic acid (BA, 3β-hydroxylup-20(29)-en-28-oic acid), and ursolic acid (UA, 3β-hydroxyurs-12-en-28-oic acid) (Figure 1) are bioactive pentacyclic triterpenoids present in natural plants in the form of free acid or aglycones for triterpenoid saponins. OA has been isolated from more than 120 plant species[1,2] and is also present in several foods as virgin olive oil.[3,4] Besides their antioxidant activity *in vitro*,[5] these pentacyclic triterpenes are very interesting for other pharmacological properties with beneficial health effects.

Recently, some supplements, such as triterpenes, including UA, BA, and OA, have been reported to have antineoplastic effects and to reinforce the activity of anticancer drugs against several tumor cell lines.[6] OA significantly enhanced insulin secretion at basal and stimulatory glucose concentrations in insulinoma-derived INS-1 (832/13) β cells and enhanced acute glucose-stimulated insulin secretion in isolated rat islets.[7] Also, the administration of the natural triterpenoid OA reduces and limits the severity and development of experimental autoimmune encephalomyelitis. Therefore, OA therapy might be of clinical interest for human multiple sclerosis and other Th1 cell-mediated inflammatory diseases.[8] In accordance with Kashiwada et al.,[9] there is evidence of the antiviral effects, including anti-human immunodeficiency virus (HIV). Furthermore, UA might be a useful anticancer drug in treatment of human papilloma virus (HPV)-associated cervical neoplasia.[10] Ramachandran and Prasad[11] conclude that UA is a dietary antioxidant on lipid peroxidation induced by ultraviolet (UV) radiation from 290 to 320 nm (UVB). This terpenic compound also presents an antioxidant effect on oxidative DNA damage, with reference to alterations in cellular antioxidant status in human lymphocytes. Horiuchi et al. tested antimicrobial activity of similar triterpenoids, OA, uvaol, BA, betulin, and UA. OA and UA showed antimicrobial activity against *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA). The antimicrobial activity of OA or UA is not so strong as compared to antimicrobial drugs that are clinically used, although OA and UA showed fairly high activity.[12] Nonetheless, it seems that both compounds are not so toxic. In fact, OA has been successfully used as an orally administrated drug to treat human liver diseases in China.[13] According to Reisman et al., a study in mice demonstrates that OA facilitates Nrf2 nuclear accumulation, causing induction of Nrf2-dependent genes, which contributes to protection from acetaminophen hepatotoxicity.[14] Lin et al.,[15] in a hamster trial, conclude that OA and UA are responsible for the cholesterol-lowering effect of hawthorn by inhibiting intestinal acylCoA:cholesterol acyltransferase activity. In addition, hawthorn and, particularly, its bioactive compounds (OA and UA) enhanced the cholesterol-lowering effect of plant sterols. Recently, Liu et al. examined the therapeutic effects of pentacyclic triterpenes, OA and MA, on hyperlipidemia in a high-cholesterol diet model. Triterpene treatment possesses therapeutic effects on diet-induced hyperlipidemia by inhibiting the intestinal absorption and storage of cholesterol.[16]

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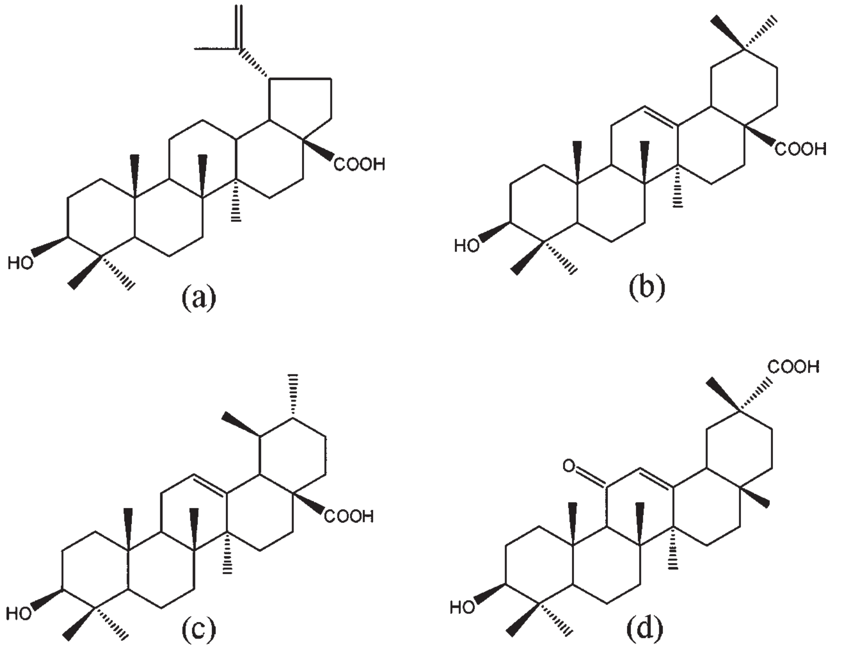

Figure 1. Chemical structures of triterpenic compounds determined in human serum: (a) BA, (b) OA, and (c) UA and (d) glycyrrhetinic acid (internal standard).

In recent years, it has been found that OA inhibits DNA synthesis and induces apoptotic events in human astrocytoma cells;[17] beside this, OA contributes to vascular homeostasis by inducing PGI2 release in a Cox-2-dependent manner.[18] On the other hand, BA acts as a novel anticancer agent through targeted degradation of Sp proteins that are highly overexpressed in tumors.[19] UA rarely occurs without its isomer OA, having similar pharmacological activity,[20] making them useful in cosmetic applications as well. UA has been used to treat photoaged skin because it prevents and improves the appearance of wrinkles and age spots by restoring the collagen bundle structures of the skin and its elasticity.[21−23] Research has found BA to be particularly useful against melanoma, a dangerous form of skin cancer, by targeting tumors without destroying healthy cells.[24,25]

Mixtures of these triterpenoids, as well as their conjugates with natural sugars, are known as gerontoprotective (hypocholesterolemic, cardiotonic, and anti-atherosclerotic[26]), and other types of health-improving bioactivities.[27] In the analysis of triterpenoids, it is necessary to use a technique with improved analytical recovery, better resolution, and greater sensitivity. At present, works that analyze these compounds in human biological samples are very scarce. Song et al. determine OA in human serum by high-performance liquid chromatography (HPLC)/mass spectrometry (MS).[28] Our research was focused on OA, BA, and UA. The aim of this work was to show how, by employing the HPLC technique, we can achieve separation, identification, and quantification of pentacyclic triterpenoids in complex human samples, suitable for nutritional and pharmacological studies.

Although it is important to understand the contribution of each isolated plasma protein to drug binding, the data obtained with individual components of plasma are not applicable to the clinical situation. For this reason, the interactions between OA or BA and human serum proteins (HSPs) were studied to understand the differences in their biological activities.

■ EXPERIMENTAL SECTION

Samples, Solvents, and Standards. Human plasma was collected from normal healthy volunteers. Blood samples were collected in Vacuette tubes (5 mL, Z Serum Sep Clot Activator) and centrifuged for 15 min at 3000*g* at room temperature. The serum samples were stored at −80 °C until analysis. All reagents and solvents used were of HPLC grade. OA (purity 97%), UA (purity 90%), BA (purity 90%), glycyrrhetinic acid (purity 98%), and cholesterol (purity 99%) were obtained from Sigma-Aldrich (Germany). Methanol, ethyl ethanoate, diethyl ether, dichloromethane, hexane, acetonitrile, acetic acid, formic acid, and phosphoric acid were supplied by Panreac (Spain). OA employed in this study was obtained in our laboratory from olive leaves in accordance with the procedure described by Guinda et al.[29]

HPLC Determination. *Sample Preparation.* To 3 mL of serum sample, a methanolic solution of 18β-glycyrrhetinic acid (2.5 μg/mL) [internal standard (IS)] was added. The mixture was extracted in triplicate with 5.0 mL of diethyl ether through vigorous vortex mixing for 3 min. The two phases were separated by centrifugation at 2500*g* for 7 min. The supernatant diethyl ether layer was separated and completely evaporated at 40 °C under a gentle stream of nitrogen gas. The dry residue was reconstituted in 100 μL of acetonitrile/0.5% aqueous phosphoric acid (85:15, v/v) mobile phase, vortex mixed for 30 s, and filtered through a 0.45 μm syringe filter prior to HPLC. A total of 20 μL was injected onto the HPLC column.

HPLC Separation−Detection. HPLC was performed with a Waters 600 series HPLC system with a photodiode array detector, equipped with a Rheodyne injection valve (20 μL, loop) and data acquisition system. Compounds were separated on a 30 °C thermostatized C_{18} reversed-phase Waters Spherisorb ODS-2 column (250 × 4.6 mm inner diameter, 5 μm particle) (Sigma-Aldrich, Spain). The analyte was eluted isocratically at a flow rate of 0.8 mL/min with the mobile phase of acetonitrile and 0.5% phosphoric acid in water (85:15, v/v). The optimum wavelength for determination was 204 nm.

Calibration Curve. Stock solutions of the triterpenic acids (TAs) (OA, BA, and UA) and the IS, glycyrrhetinic acid, were prepared at a concentration of 50 μg/mL in methanol. The solutions were stored

Table 1. Recovery and Precision Data of Pentacyclic Triterpenoid Analysis by HPLC (Diethyl Ether Liquid Extraction)[a]

	OA			BA			UA		
sample	mean (ng/mL)	RSD[b]	recovery (%)	mean (ng/mL)	RSD	recovery (%)	mean (ng/mL)	RSD	recovery (%)
1	152.4	4.8	95.2	158.6	5.1	94.0	147.3	4.7	95.6
2	117.0	5.0	96.4	120.1	5.7	93.6	121.4	5.7	94.5
3	88.4	6.2	94.5	92.2	6.5	94.2	90.5	6.9	93.8
4	42.0	7.9	93.7	44.8	8.1	92.7	43.6	8.0	94.1
5	20.8	8.5	94.3	23.0	8.9	93.1	22.0	9.3	93.5
6	9.3	9.7	92.1	11.4	10.0	92.4	10.3	9.9	92.7

[a] $n = 6$, with four sample replicates and duplicate injections for each sample. [b] RSD = relative standard deviation (%).

at 4 °C and showed no significant alterations in peak areas or heights determined daily by direct injection throughout the study. Working standard solutions (20.0, 50.0, 100.0, and 500.0 ng/mL) of TAs were prepared by serial dilution with the same solvent.

The blank serum samples were spiked with TAs in concentrations of 10, 20, 40, and 100 ng/mL, respectively. Once the triterpene compounds was incorporated into the serum at room temperature by agitation for 10 min, each spiked serum sample (3 mL) was put through the complete sample procedure, including the addition of 20 μL of IS, as described above. The resulting sample solution was subjected to HPLC analysis.

Recovery from Human Serum. Linear calibration graphs were obtained from 25 to 1000 ng/mL for the triterpenoid standards at concentrations of 50, 400, and 800 ng/mL. Recovery of IS from human serum was determined at the concentration used in the samples. The recoveries were measured by a direct comparison of peak-area ratios obtained for non-extracted and extracted standards from human serum.

Interaction of TAs with HSPs. The stock solutions of OA and BA were prepared by dissolving the respective terpenic acid in methanol to yield 1 mg/mL stock solutions. The last solutions were used for the preparation of standards and samples for both plasma protein binding. Appropriate amounts of OA methanol stock solution were taken, evaporated to dryness under nitrogen, and reconstituted in blank plasma to give human serum protein−oleanolic acid (HSP−OA) system samples at 10.96, 39.42, and 60.22 μM, then allowing equilibration to take place for 15 min at 37 °C prior to spectrophotometric analysis. The human serum protein−betulinic acid (HSP−BA) standards were prepared in a manner similar to that described for HSP−OA samples to yield a series of standards ranging from 54.75 to 60.22 μM. To obtain the visible spectrum of triterpenoids, the samples were prepared in aqueous solution. Deionized water with Milli-Q Integral system (Millipore) was spiked with appropriate amounts of TAs stock solutions to give samples at 10.96 and 5.48 μM of OA and BA, respectively. All samples were made in triplicate. The spectrophotometer curves were generated by analyzing the standard samples immediately after they were made. The absorption spectra were recorded (240−340 nm) on a microfocused beam Beckman Coulter DU 800 ultraviolet−visible (UV−vis) spectrophotometer equipped with a LH-72 D2 lamp and a bandwidth of 1.8 nm. A quartz cell of 1.00 cm with a temperature maintained at 20 ± 0.1 °C was used for measurements.

■ RESULTS AND DISCUSSION

Optimization of the Extraction Procedure. Several extraction methods, solvents, and times were investigated to obtain the best extraction efficiency. Various solvents, including dichloromethane, hexane, diethyl ether, ethyl ethanoate, and their mixtures in different combinations and ratios, were screened (data not shown), and the best solvent was found to be diethyl ether, which enabled less interfering peaks and provided the highest values in the contents of the four markers (Table 1), extracting >90%. On the other hand, hexane showed the worst behavior because it extracted less than 40% of the TAs. The volume of diethyl ether, times of extraction, and duration of the same were also investigated to optimize the extraction procedure. The results demonstrated that the established extraction method (each sample was extracted 3 times by vigorous vortex mixing with 5 mL of diethyl ether for 3 min per time) was adequate and appropriate for the analysis.

Optimization of the Chromatographic Conditions. To obtain chromatograms with better resolution of adjacent peaks within shorter time, the chromatographic conditions were optimized. The resolutions of these compounds were tested and compared to different reversed-phase conditions using a variety of analytical columns, such as Agilent Lichrospher C18 (250 × 4.6 mm, 5 μm) and Waters Spherisorb ODS-2 C_{18} (250 × 4.6 mm, 5 μm). Considering most of the compounds under study have a wavelength maximum at 204 nm, to avoid blank interference, acetonitrile was chosen as the organic solvent because of its low absorbance compared to methanol. Because all reference compounds are TAs, the investigation is commenced with a buffer at acidic pH to improve the resolution and eliminate the tailing of the target peak. Hence, 0.1% formic acid, 0.4% acetic acid, and 0.5% phosphoric acid were compared in the experiment. As a result, a Waters Spherisorb ODS-2 C18 (250 × 4.6 mm, 5 μm) column with acetonitrile and 0.5% aqueous phosphoric acid (85:15, v/v) as the mobile phase were chosen as the preferred chromatographic conditions and isocratic elution was applied. It was also suggested that separation was better when the column temperature was kept at 30 °C rather than 25 and 40 °C. According to the absorption maxima of the reference compounds on the UV spectra with three-dimensional chromatograms of HPLC−diode array detection (DAD), the wavelength was set at 204 nm for TAs and 254 nm for glycyrrhetinic acid. Representative chromatograms for the standard analytes and for a sample are shown in Figure 2. The chromatographic peaks were identified by comparing their retention time to that of each reference compound, which was eluted in parallel with the optimized mobile phase. All compounds can be well-distinguished by order of elution during liquid chromatography. In addition, the serum was screened for interference at the retention times of TAs and glycyrrhetinic acid. No endogenous compounds occur at the retention times of TAs nor at IS retention time (panels b and c of Figure 2).

Validation of the HPLC Method. *Calibration Curves and Limits of Detection (LD) and Quantification (LQ).* The stock solution containing the four markers was prepared and diluted to six appropriate concentrations for the establishment of calibration curves. The calibration graphs were plotted after linear regression of the peak areas versus the corresponding

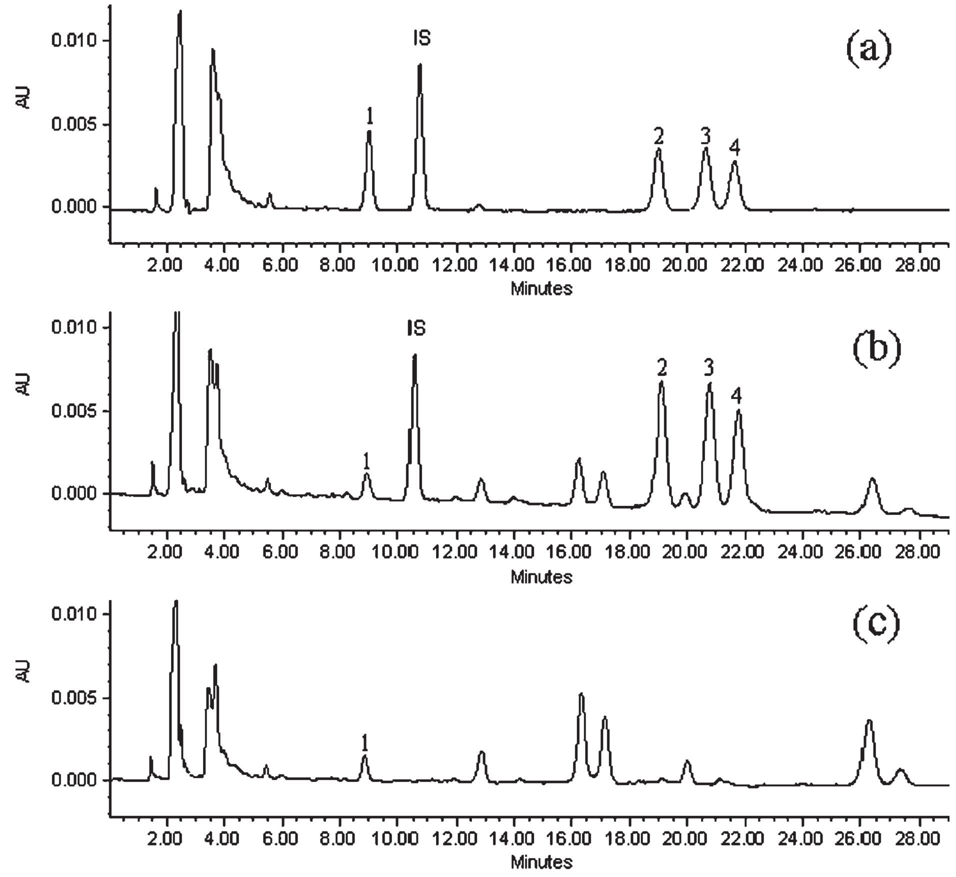

Figure 2. Typical HPLC chromatograms of (a) mixed standards and (b) serum spiked with glycyrrhetinic acid (IS), OA (2), BA (3), and UA (4), at highest concentrations; cholesterol (1) belongs to blank serum. (c) Blank serum (4 mL).

concentrations. The lowest concentration of working solution was diluted with methanol to a series of appropriate concentrations, and aliquots of diluted solutions were injected into HPLC for analysis. The LD and LQ for each analyte under the chromatographic conditions were determined at the signal-to-noise ratio (S/N) of 3 and 10, respectively. The results of the *F* test for comparison of the variance of residuals and the variance of the regression indicated that all reference compounds showed good linearity ($p < 0.01$; $r^2 > 0.997$) in a relatively wide concentration range. The LD and LQ of the analytes were 3.3−4.3 and 11.0−14.3 ng/mL, respectively.

Precision, Repeatability, and Stability. The intra- and interday precisions were investigated by determining the four analytes in six replicates during a single day and duplicating the experiments on 3 consecutive days. Repeatability was confirmed with six different working solutions prepared, and one of them was injected into the apparatus at 0, 4, 8, 12, 24, and 48 h within 2 days, respectively, to evaluate the stability of the solution. Variations were expressed by relative standard deviations (RSDs). The intraday coefficients of variation for the measurement of TAs in human serum ranged from 3.3 to 6.4% depending upon the concentration level. The interday coefficients of variation ranged from 4.9 to 9.1%. These low coefficients of variation indicate the good precision of the established HPLC assay.

Recovery. The recovery was performed by adding known amounts of the standards at low (80% of the known amounts), medium (same as the known amounts), and high (120% of the known amounts) levels. The spiked samples were then extracted, processed, and quantified in accordance with the method mentioned above. The results were shown in Table 1. The overall recoveries lay between 92.1 and 96.4%, for all reference compounds, with RSD less than 10.0%, and the recoveries are comparable to 100% at 95% confidence level ($p > 0.05$), which indicated that the established method was accurate enough for the determination of these TAs in human serum.

UV−Vis Absorption Spectroscopy. Proteins present intrinsic absorbance at approximately 280 nm. Minor HSP (0.5−1 mg/mL of plasma) plays an important role in the binding of basic and neutral drugs. In contrast, the most abundant plasma protein, human serum albumin (HSA), is present in much higher concentrations, ranging from 35 to 45 mg/mL of plasma. HSA is responsible for binding mainly acidic drugs.[30] On the basis of these considerations, we made the hypothesis of the possible formation of terpenic acid serum albumin complexes.

The molecular interactions are often monitored by spectroscopic techniques because these methods are sensitive and relatively easy to use. They have advantages over conventional approaches, such as affinity and size-exclusion chromatography, ultrafiltration, and ultracentrifugation, which suffer from the lack of sensitivity, long analysis time, or both. In the present paper, we report an interaction study of TAs with HSA by the UV spectrometry method.

Absorption spectra in the UV region measurement is an applicable method to explore the structural change and to know the complex formation.[31] In the present study, we have observed the change in UV absorption spectra of HSP−OA and HSP−BA systems.

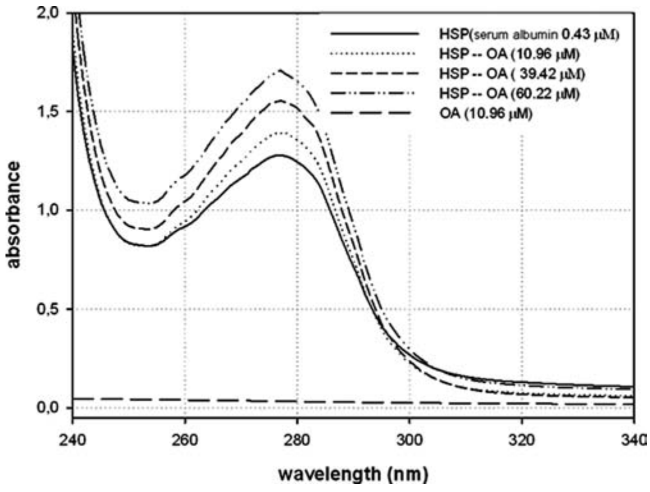

Figure 3. Absorption spectra of HSP, OA, and the HSP−OA system in 0.1 M phosphate buffer at pH 7.2. The HSP concentration is 0.43 μM. The OA concentration for the HSP−OA system is at 10.96, 39.42, and 60.22 μM. A concentration of 10.96 μM is used for OA only.

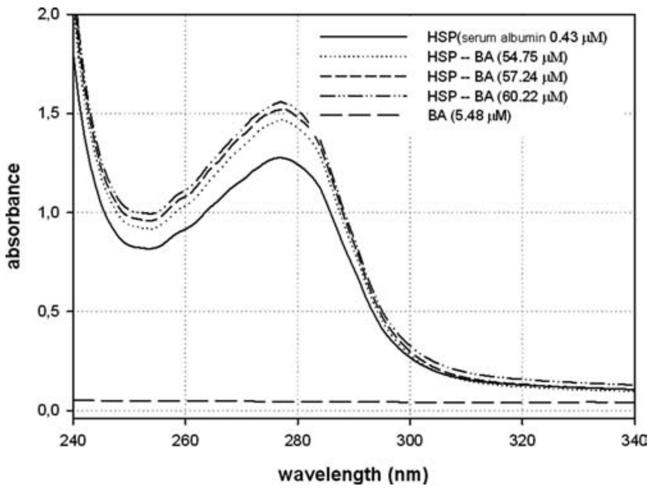

Figure 4. Absorption spectra of HSP, BA, and the HSP−BA system. The HSP concentration is 0.43 μM. The BA concentration for the HSP−BA system is at 54.75, 57.14, and 60.22 μM. A concentration of 5.48 μM is used for BA only.

Figures 3 and 4 show the spectrum from 240 to 340 nm, with maximum absorption at 278 nm of 0.43 μM HSP, expressed as serum albumin (continuous line). It is evident that the addition of OA increases the absorption intensity of HSP at $\lambda_{\max}$ (Figure 3), indicating a change in the protein conformation and, hence, the HSP−OA system formation.[32]

The HSP−BA system behavior was similar to the HSP−OA system (Figure 4); the absorption intensity at $\lambda_{\max}$ of HSA is increased regularly with the addition of BA, although a greater absorbance increase at $\lambda_{\max}$ is observed for the HSP−OA system at the same triterpenoid concentration (60.22 μM). The spectrum recorded profound hyperchromic effects by the addition of OA and BA with a 34.9 and 13.8% increase in absorbance of the initial value, respectively. This observation is attributable to the intercalation of TA with HSP. The minor differences between absorbancies at 278 nm of HSP−BA systems are due to minor differences in the concentrations of BA in the human serum. The UV−vis spectrum (Figures 3 and 4) of OA and BA do not show any shift of $\lambda_{\max}$.

In conclusion, a simple and reproducible HPLC method has been developed to determine simultaneously the TAs, OA, BA, and UA, in human serum samples, using a Waters Spherisorb ODS-2 C18 reversed-phase column. The quantitative ranges were 11.0−14.3 ng/mL and the intra- and interday coefficients of variation were satisfactory for the determination of the studied triterpenoids in serum. We conclude that the established HPLC method can be suitable for pharmacokinetic studies in humans after ingestion of a single dose of drugs and may be recommended for monitoring these compounds in patients. In comparison to the related method by Song et al.,[28] this HPLC assay determines simultaneously OA, UA, and BA because, from a biological point of view, they are the most important triterpenoid structures (oleanane, ursane, and lupane triterpenoids). In addition, this paper provides an approach for studying the interactions of HSP with TAs using UV absorption spectrometry. The results showed the complex formation between HSP and OA and BA. The biological significance of this work is evident because serum protein serves as a carrier molecule for multiple bioactive compounds and the interaction of TAs with HSP has not been reported thus far. This report has great significance in nutrition research to study the metabolic pathway of TAs, as well as methodology.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mrada@ig.csic.es.

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■ ABBREVIATIONS USED

BA, betulinic acid; HIV, human immunodeficiency virus; HPV, human papilloma virus; HSP, human serum protein; IS, internal standard; OA, oleanolic acid; TA, triterpenic acid; UA, ursolic acid; UV−vis, ultraviolet−visible

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