Title: RP-HPLC method development for the simultaneous determination of timolol maleate and human serum albumin in albumin nanoparticles

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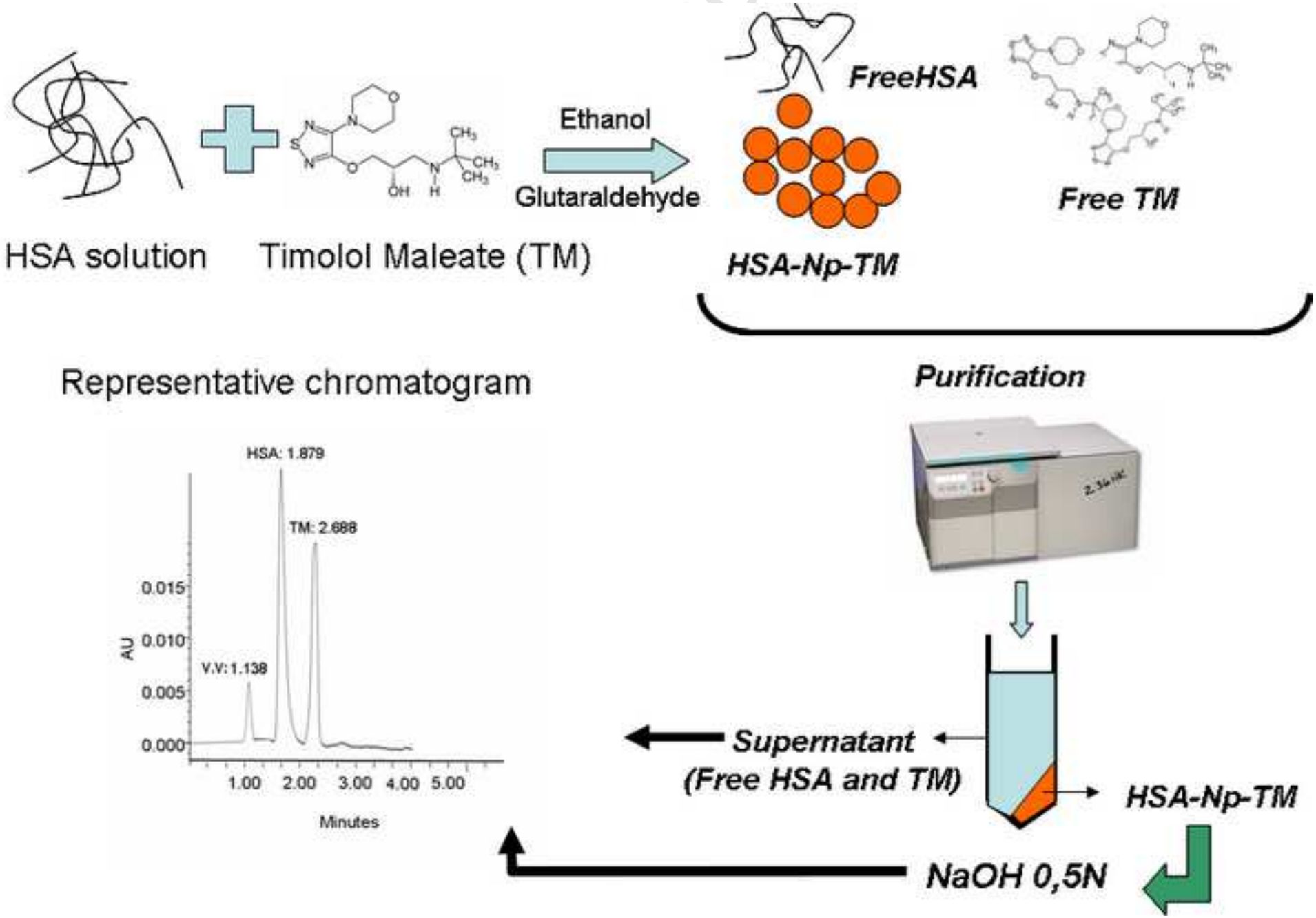
*Graphical Abstract



Highlights

- New HPLC method for the simultaneous determination of HSA and TM in nanoparticles.
- Rapid, reproducible and selective reversed phase HPLC method with UV detection.
- Useful for quantification of process yield and for efficiency of encapsulation of timolol maleate and human serum albumin nanoparticles.

RP-HPLC method development for the simultaneous determination of timolol maleate and human serum albumin in albumin nanoparticles

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Abstract

An isocratic high-performance liquid chromatographic method was developed and validated for the simultaneous determination of human serum albumin (HSA) and timolol in albumin nanoparticles. This method involved a reversed-phase-C18 column thermostated at 25 ºC, UV detection at 276 nm, flow rate of 1.0 ml/min and a mobile phase compounded by 0.05% (v/v) trifluoroacetic acid in water/0.05% (v/v) trifluoroacetic acid in an acetonitrile (40:60 v/v) solution. The elution times for albumin and timolol were 1.84 ± 0.05 min and 2.67 ± 0.04 min, respectively. Calibration curves were linear from 0.2-100 mg/ml for HSA and 0.01-1mg/ml for timolol. Limits of quantification were 0.2 mg/ml for HSA and 0.01 mg/ml for timolol. The values of accuracy and precision of intra- and inter-day variation studies were within acceptable limits, according to the US Food and Drug Administration Guidance for Industry. The described method has proved to be useful to give accurate measurements of human serum albumin and timolol from albumin nanoparticles to determine the percentage of encapsulation and the process yield.

Keywords: Human serum albumin; Timolol Maleate; Protein Nanoparticles; HPLC; Validation

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1. Introduction

Topical application onto the eye´s surface is the most common route for drug administration to treat ocular diseases. However, the protective mechanisms of the eye (blinking, drainage, baseline and reflex lachrymation) decrease the bioavailability of drugs by rapidly removing foreign substances such as drugs, dust particles and bacteria. This is the main reason why it is necessary to optimize a pharmaceutical carrier to overcome these drawbacks [1].

Drugs administered by means of conventional ophthalmic formulations usually show poor bioavailability. Such formulations are usually aqueous solutions, suspensions or ointments. Unfortunately, only 5% of the administered drug is able to reach the intraocular tissues [2]. This is a consequence of fast drainage from the precorneal area. Besides, high amounts of the drug may be systemically absorbed both by the conjunctiva and the nasolacrimal duct [3-5]. In addition, the cornea is practically impermeable and behaves like a very efficient barrier against chemical compounds.

Ocular bioavailability may be enhanced by means of two strategies: i) increasing the contact time of the drug with the eye surface and ii) promoting the transfer of drug molecules from the tear into the eye.

Nanoparticles are colloidal drug-carrier systems, in which drugs are either dissolved, entrapped or encapsulated [6]. These pharmaceutical systems may provide sustained drug release and prolonged therapeutic effect [**7**].

The characterization of nanoparticles involves measurements of particle size, zeta potential and other determinations such as process yield and encapsulation efficiency [8, 9]. Specifically, it is necessary for human serum albumin (HSA) nanoparticles to determine at the same time both albumin and drug aiming to evaluate process yield as well as encapsulation efficiency. In some cases, however, the simultaneous determination of both components becomes very complicated and

somewhat dubious, leading researchers to carry out indirect measures for the correct quantification of the analytes.

With this in mind, we aimed our research mainly at the development of a formulation based on HSA nanoparticles (HSA-Np) containing timolol maleate (TM), intended to overcome the difficulties mentioned above for the treatment of chronic eye diseases like glaucoma. It was hypothesized that due to size and muchoadhesive properties, such nanoparticles may better interact with ocular epithelium and may deliver the drug on to the ocular surface with longer residence time.

Although each of the mentioned compounds has been extensively studied and information about them can be found in the literature [10-15], no method for the simultaneous determination of HSA and TM has been reported up to the present day.

Consequently, and in order to ensure the availability of the required analytical tools for the simultaneous quantitative determination of the main components of the formulation (HSA and TM), it was necessary to develop and validate a suitable chromatographic method.

The development of this method presents a number of advantages. In addition to short analysis time and reduced costs (the compounds of interest are determined by a single chromatographic run in a relatively short time), it is also a direct measurement technique (previous steps of an indirect determination are not required) able to avoid errors in the measurement and / or to prevent contamination of the analytes since it involves a single determination without previous steps (the sample of interest must be processed only once).

The novel method reported here was validated according to standard guidelines [16] and was found to be accurate, fast and economical for the simultaneous determination of TM and HSA

2. Materials and methods

2.1. Materials

Human serum albumin (HSA) and glutaraldehyde (25% solution) were supplied by Sigma-Aldrich (Buenos Aires, Argentina) and timolol maleate (TM) was purchased from Parafarm (Buenos Aires, Argentina). Trifluoroacetic acid and acetonitrile (HPLC-grade) were purchased from Sintorgan (Buenos Aires, Argentina). Milli-Q water was used in all experiments.

2.2 Methods

2.2.1 Nanoparticles preparation

Human serum albumin nanoparticles (HSA-Np) were prepared by a desolvation method by addition of ethanol to a 2% solution of HSA (1:2) under continuous stirring. Then, coacervates were hardened by crosslinking with glutaraldehyde (1.56 μg/mg protein) for 5 hs. Next, the ethanol was eliminated by evaporation under reduced pressure (Rotavapor R110, BÜCHI Labortechnik, Flawil, Switzerland). For the preparation of timolol nanoparticles (TM-Nps) the method used was the same as the described previously, except that 5 mg the drug were added to the albumin solution before adding the desolvation agent. The resulting nanoparticles were purified by centrifugation at 21000 rpm for 20 min (HERMLE Z 36 HK, Labortechnik) to eliminate both free albumin and drug. Then, the pellets were dispersed to the original volume in water and the supernatant was removed. For the determination of particle size, polidispersity index and zeta potential, the samples were diluted in purified water and measured at a temperature of 25°C and a scattering angle of 90° in DelsaNano-C (Beckman Coulter, Osaka, Japan) with software of DelsaNano 2.20 (TM)

2.2.2 Chromatographic system

The chromatographic system consisted of a Waters 1525 pump, a Waters 717 plus autosampler, a Waters 1500 series column heater and a Waters 2996 photo array detector (PDA) (Waters Corp., Milford, USA). The wavelength was set at 276 nm.

Data acquisition was performed by the Empower Software(TM) data registration. The analytical column was a reversed-phase Luna C18 (250 × 4.6 mm i.d., 10 µm particle size, Phenomenex, Torrance, California, USA) maintained in the column oven at 25°C and protected by a Phenomenex (TM) Security Guard precolumn. The mobile phase consisted of 0.05% (v/v) trifluoro acetic acid in water: 0.05% (v/v) trifluoroacetic acid in acetonitrile (40:60 v/v). The elution was carried out isocratically at a flow-rate of 1 ml/min. The mobile phase was filtered through a 0.45 µm Millipore Durapore(TM) (Billerica, Massachusetts, USA) filter and degassed by vacuum prior to use.

2.3. Preparation of calibration standards and quality control (QC) samples

Stock standard solutions of HSA and TM were prepared by weighing out appropriate amounts of each component ($1x10^3$ mg of HSA and 50 mg of TM) and dissolving them in 50 ml of water. Standard solutions and QC samples were prepared by a serial dilution of primary stock solutions using purified water. The calibration standards of 0.2, 1.0, 2.5, 10, 15 and 20 mg/ml; those of 0.01, 0.05, 0.1, 0.3, 0.6 and those of 1.0 mg/ml were prepared for HSA and TM, respectively. QC samples at three different levels (0.2, 10 and 20 mg/ml for HSA; and 0.01, 0.3 and 1.0 mg/ml for TM) were fixed daily from their corresponding stock solutions.

2.4 Validation parameters

2.4.1 Calibration curves

Calibration curves were constructed by triplicating at 6 and 7 concentration levels from the standard solutions of HSA and TM, respectively, and analyzing them for

two days. The data of the peak area versus concentration were treated by linear least square regression analysis. Selectivity, lower limit of quantitation (LOQ), and method accuracy and precision were assessed by using some of these standard solutions.

The last two parameters were determined by analyzing six replicates of QC samples at three concentration levels at high, medium and low concentration ranges of the calibration curves for each analyte and then comparing these with their theoretical concentrations. With the same purpose, the interference of the analytes (HSA and TM) between them was evaluated by means of injections of standard solutions of each analyte and by the addition of the other analyte as interference at a different concentration. The precision of the method was assessed by the relative standard deviation (RSD %) values of the results that corresponded to the peak area. These values were expressed for intra-day precision and on 2 days for intermediate (inter-day) precision.

3. Results and discussion

3.1. Method development and optimization

The analytical method was developed from that proposed by Rele et al. for the determination of Latanoprost and TM in combined pharmaceutical dosage forms [13]. Both the specified mobile phase and flow-rate were satisfactory since the tailing factors of both HSA and TM were 0.8 and 1.36, respectively. These values were within the acceptable limit, resulting in good peak symmetry and resolution. Under these conditions, very acceptable retention times of around 1.84 ± 0.05 min for HSA and 2.67 ± 0.04 min for TM were obtained. A representative chromatogram corresponding to both analytes is shown in Fig. 1.

INSERT FIGURE 1

The selectivity of the method was also tested by observing potential interferences between HSA and TM. No peaks interfering among themselves were observed in the chromatograms.

3.2 Linearity

The calibration curves were linear over the studied concentration ranges. The results are presented in Tables 1 and 2 and showed good correlation between the peak area of analytes and a concentration with $r^2 > 0.9974$ for all curves.

INSERT TABLE 1

INSERT TABLE 2

3.3. Precision, accuracy and LOQ

Precision and accuracy values calculated for the QC samples during the intra- and inter-day run are given in Table 3.

INSERT TABLE 3

The accuracy of the assay method refers to the closeness between the mean of the measured values and their true values. From Table 3 it can be concluded that the accuracy values in intra and inter-day variation studies at low, medium and high concentrations for HSA and TM fall within the acceptable limits of 98% and 102 %.

The accuracy of the proposed method was also verified by means of recovery assays for HSA and TM in the synthetic admixtures of both drugs. Three successive determinations of each solution were carried out and the percentage of recovery was calculated in each case. The results obtained from the recovery of both drugs (table 3) showed very good accuracy.

Precision, expressed as the relative standard deviation percentage (RSD%) of replicates, is a measure of the relative errors of the method. In this work, precision was tested both by intra- and inter-day repeatabilities at the three QC standards that cover the assay method range. In all cases, RSD% was lower than 3.7%, suggesting adequate repeatability of the assay method.

The LOQ was considered in this work as the lowest concentration standard granting acceptable accuracy and precision. The LOQ of the method was found to be 0.2 mg/ml for HSA and 0.01 mg/ml for TM, with an accuracy of 98-102 % and RSD% values between 0.33 and 3.72.

3.4 Selectivity

The selectivity of the HPLC method is illustrated in Fig. 1 where a complete separation of HSA and TM can be observed. The analyte peaks are narrow and there is no interference between them, thus confirming the selectivity of the analytical method.

4. Applications

For this work, HSA-Np were prepared by a desolvation method [17] with the addition of ethanol to a HSA solution (1:2) and crosslinking them with glutaraldehyde. Solid NPs thus obtained were resuspended in an aqueous solution containing 5% saccharose, which was added as cryoprotector for the further process of freeze-drying.

The yield was calculated through two different methods. On the one hand, the nanoparticle obtained after lyophilization was digested with NaOH 0.5N at room temperature, under magnetic stirring for 1 h. The resulting solutions were then measured in HPLC (direct measurement). On the other hand, the supernatants collected from centrifugation were analyzed by HPLC (indirect measurement) where the amount of HSA and TM into nanoparticles was calculated by the difference between the total

amount of HSA or TM initially added and the amount determined in the supernatants. Table 4 shows the results obtained.

INSERT TABLE 4

5. Conclusions

This paper describes a rapid and reproducible HPLC method which enables the simultaneous determination of HSA and TM for the quantification of process yield and encapsulation efficiency for NPs formulations. The typical assay time is about 5 min. The relatively short retention times for HSA and TM in our technique makes it possible to analyse a large number of samples over a short period of time. In conclusion, the HPLC method developed using UV detection shows good selectivity and is suitable for a reliable determination of these compounds. The HPLC assay method presented here has been successfully applied to the evaluation of the pharmaceutical performance with potential applicability in the treatment of glaucoma.

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Figure Caption

Figure 1: Representative HPLC-UV chromatograms from Void Volume (V.V), human serum albumin (HSA) and timolol maleate (TM)

Table 1: Results of regression analysis of HSA linearity data

y = bx + a	C1*	C2*	C3*	Mean ± SD
a (intercept)	-16461	-21229	-35161	-24284±9717
b (slope)	681906	690466	674677	682350±7908
r^2 (determination coefficient)	0.9994	0.9974	0.9999	0.999±0.001

*Replications of calibration curves

Table 2. Results of regression analysis of TM linearity data

y = bx + a	C1*	C2*	C3*	Mean ± SD
a (intercept)	-3108	-8796	-4455	-3510±824
b (slope)	12100000	12600000	12600000	$(12.4\pm 0.2)\ 10^6$
r^2 (determination coefficient)	0.9999	0.9999	0.9996	0.9998 ± 0.0002

* Replications of calibration curves

343

344 **Table 3. Precision and accuracy of the HPLC assay for HSA and TM**

345

Nominal concentration (mg/mL)	Calculated concentration (mean ± SD) (mg/mL)	Precision (RSD %)	Accuracy (% recovery)	N
Day 1				
HSA				
0.2	0.196±0.002	1.27	98.00	3
10	10.0±0.4	3.72	100.30	3
20	20.2±0.2	1.09	101.02	3
HSA + (TM 0.497mg/mL)				
0.2	0.198±0.002	1.01	99.00	3
10	10.12±0.09	0.86	101.20	3
20	20.1±0.3	1.34	100.75	3
TM				
0.01	0.0102±0.0002	1.02	102.00	3
0.3	0.298±0.002	0.67	99.33	3
1	0.99±0.01	1.50	99.30	3
TM (HSA 10mg/mL)				
0.01	0.0101±0.0003	2.90	101.00	2
0.3	0.302±0.002	0.66	100.70	2
1	1.005±0.014	1.39	100.50	2
Day 2				
HAS				
0.2	0.199 ±0.006	3.00	99.50	3
10	9.9±0.2	1.70	98.80	3
20	19.9±0.4	2.20	99.45	3
TM				
0.01	0.0102±0,0001	0.68	102.00	3
0.3	0.298±0.001	0.33	99.33	3
1	1.01±0.01	1.20	100.70	3

346 S.D.: standard deviation; N: number of replicates.

347 **Table 4: Physico-chemical characteristics of HSA nanoparticles loaded with timolol maleate**

	Size (nm)	PI	Zeta Potential (mV)	Yield ^{Sp} (%)	Yield Nps ^{D} (%)	TM encapsulated (%)
HSA-NPs	179.0±0.1	0.12±0.03	12.0 ±2.1	73.2±8.1	73.2±12.3	----
HSA-NPs-TM*	167.9±7.2	0.15±0.03	10.5±1.6	86.3±7.2	71.9±9.1	32.5±1.2%

348

349 PI: Polydispersity index
350 Sp: Measurements obtained in supernatants
351 D: Measurements obtained by direct measurement
352 *: Nanoparticles with TM

353

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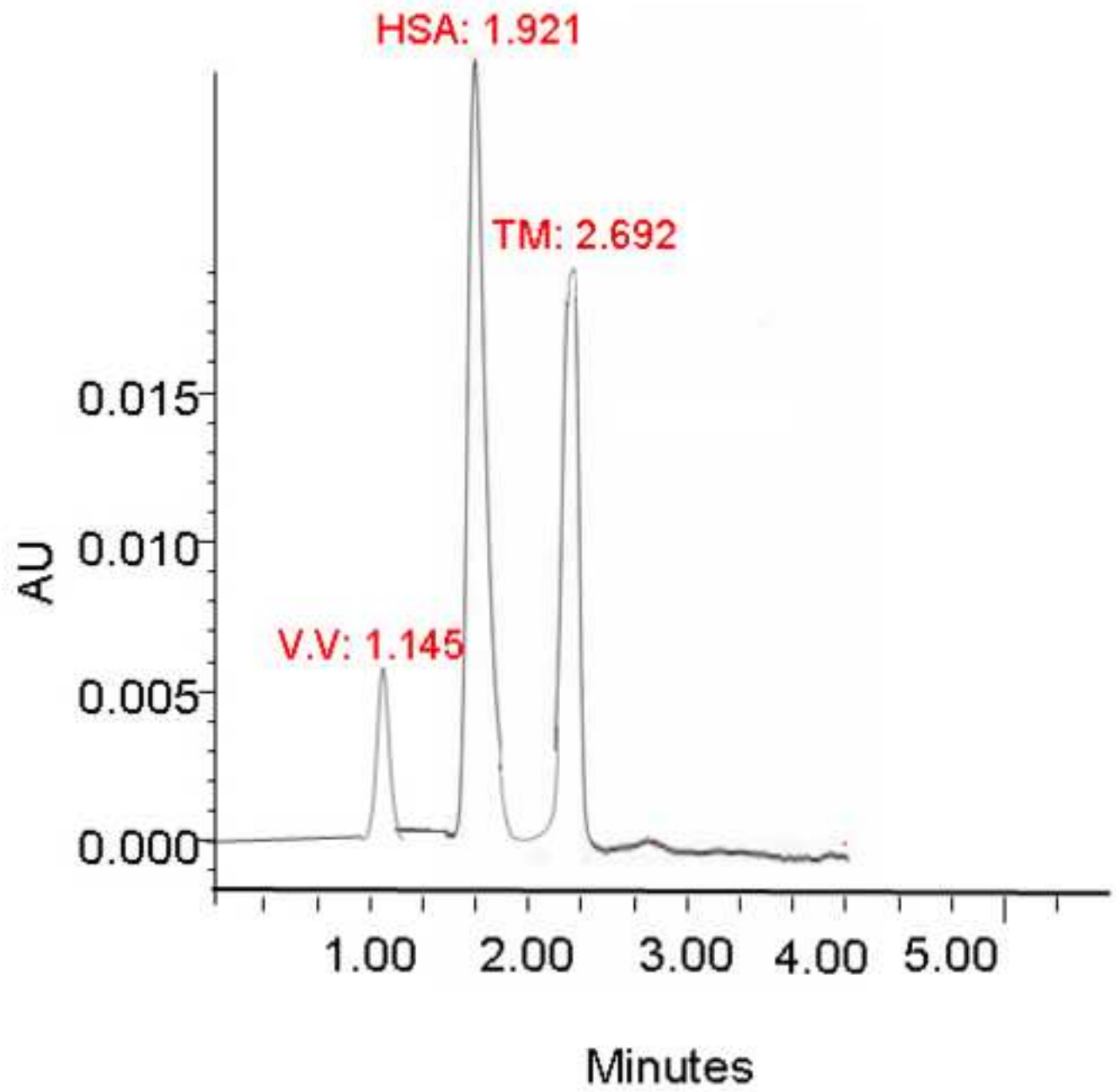
Figure(s)


HSA: 1.921
TM: 2.692
V.V: 1.145
0.015
0.010
0.005
0.000
AU
1.00
2.00
3.00
4.00
5.00
Minutes