Journal of Chromatography A, 1218 (2011) 3376–3381

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Biocompatible in-tube solid phase microextraction coupled with liquid chromatography-fluorescence detection for determination of interferon α in plasma samples

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ARTICLE INFO

Article history:
Available online 25 November 2010

Keywords:
Restricted access material
In-tube SPME
Interferon alpha$_{2a}$
Plasma sample

ABSTRACT

The present work demonstrates the successful application of automated biocompatible in-tube solid-phase microextraction coupled with liquid chromatography (in-tube SPME/LC) for determination of interferon alpha$_{2a}$ (IFN α_{2a}) in plasma samples for therapeutic drug monitoring. A restricted access material (RAM, protein-coated silica) was employed for preparation of a lab-made biocompatible in-tube SPME capillary that enables the direct injection of biological fluids as well as the simultaneous exclusion of macromolecules by chemical diffusion barrier and drug pre-concentration. The in-tube SPME variables, such as sample volume, draw/eject volume, number of draw–eject cycles, and desorption mode were optimized, to improve the sensitivity of the proposed method. The IFN α_{2a} analyses in plasma sample were carried out within 25 min (sample preparation and LC analyses). The response of the proposed method was linear over a dynamic range, from 0.06 to 3.0 MIU mL^{-1}, with correlation coefficient equal to 0.998. The interday precision of the method presented coefficient of variation lower than 8%. The proposed automated method has adequate analytical sensitivity and selectivity for determination of IFN α_{2a} in plasma samples for therapeutic drug monitoring.

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1. Introduction

Interferons (IFNs) are a family of cytokines produced in response to viral infection. They have both antiviral and antiproliferative properties as well as immunomodulatory effects, and they are classified as type I (IFN-α, -β and -ω) and type II (IFN-γ) [1]. Interferon alpha (IFN-α) is currently one of the top selling biopharmaceutical drugs for therapeutic use against hairy-cell leukemia, AIDS, and hepatitis B and C (HCV) [2]. Three types of interferon alpha, known as 2a, 2b, and n1, have been used. Among these, 2a and 2b are recombinant forms, whereas IFN-α n1, called lymphoblastoid, is natural and is produced in lymphoblast cultures [3]. Accordingly, IFN-α_{2a} is employed in the treatment of several cancers and, in combination with ribavirin, is currently the most common treatment for chronic infection with hepatitis C virus (HCV).

The current standard treatment with interferon alpha is usually both extensive and expensive, with frequent adverse events. In addition, the sustained viral response rate is far from optimal, especially in human immunodeficiency virus (HIV) co-infected patients [4]. IFN-α has a short half-life. Upon administration, the blood IFN-α level reaches peak values within 1 h and quickly declines to an undetectable level after 24 h. The terminal half-life of IFN-α varies from 3 to 8 h in patients [5]. The normally employed dose is three million units, administered subcutaneously three times a week, for six months to one year, in order to maintain a therapeutic level [3]. Drug dosage treatment extension can markedly influence viral response rates, especially for HCV. A relationship between plasma concentrations and viral response can be postulated; however, very few studies have addressed this issue [4]. The majority of methods commonly employed for interferon analysis are based on bioassay [6], immunoassay [7], isoelectric focusing (IEF) [8], and gel electrophoresis techniques [9], which require long analysis time and lack specificity as well as sensitivity.

The current assay for analysis of interferon alpha$_{2a}$ (IFN-α_{2a}) in human serum samples is ELISA (enzyme linked immuno sorbent assay). Apart from the limited availability of this assay, immunological approaches are generally costly and time-consuming to develop, and assay imprecision and matrix interferences are common issues [10].

In-tube solid-phase microextraction (in-tube SPME) is an effective solvent free sample preparation technique that makes use of a capillary column as the extraction device [11]. Organic compounds in aqueous samples are directly extracted and concentrated into the stationary phase of capillary columns by repeated draw/eject

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doi:10.1016/j.chroma.2010.11.039

cycles of the sample solution, being further transferred to the liquid chromatographic column [12,13]. Extraction of the analyte in the in-tube SPME is based on the distribution coefficient between the sample solution phase and the SPME stationary phase. A proportional relationship is obtained between the amount of analyte extracted by SPME and its initial concentration in the sample matrix [14].

In in-tube SPME, analytes are desorbed either by mobile phase flow or by aspirating desorption solvent from a second vial, which is then transferred to the LC column by mobile phase flow. The peak broadening is small, because analytes are completely desorbed before injection [13].

In-tube SPME allows for the convenient automation of the extraction process, which not only reduces the analysis time, but also provides better accuracy, precision, and sensitivity compared with manual off-line techniques [15,16].

The restricted access material (RAM) term was introduced by Desilets et al. [17]. In this sorbent, macromolecules are excluded and interact only with the outer surface of the particle support coated with hydrophilic groups, which minimizes the adsorption of matrix proteins. The RAM materials have several different structures, but their mechanism of separation is identical: a hydrophilic barrier enables the small molecules to permeate through the hydrophobic part of the stationary phase, while at the same time it excludes the macromolecules (by physical or chemical means, or a combination of both) [18].

In-tube SPME combined with restricted-access materials enables the direct injection of biological fluids and simultaneous macromolecule exclusion and drug pre-concentration [19].

The present work demonstrates the appropriate application of automated biocompatible in-tube solid-phase microextraction coupled with liquid chromatography (LC) for determination of interferon alpha$_{2a}$ in plasma samples for therapeutic drug monitoring.

2. Experimental

2.1. Reagents and analytical standards

The water used to prepare the solutions had been purified by a Milli-Q system (Millipore, Brazil). The following reagents were analytical grade: glutaraldehyde, potassium dihydrogen phosphate, sodium borohydride, and recombinant human interferon α_{2a}, which were all acquired from Merck, Darmstadt, Germany. Acetonitrile (ACN), 2-propanol, and methanol were HPLC grade (J.T. Baker, USA). Trifluoroacetic acid (TFA) was purchased from Fisher Scientific (Leics, UK). The mobile phase was previously filtered using a 47 mm diameter, 0.45 μm cellulose membrane from Millipore. Bovine serum albumin (BSA, fraction V powder, minimum 98%) was provided from SIGMA®—Oakville, Canada.

The stock standard plasma sample was prepared with reference plasma (blank plasma) spiked with interferon alpha$_{2a}$ at a concentration of 3 MIU mL^{-1}. The working standard interferon plasma samples were prepared by diluting the stock sample (3 MIU mL^{-1}) in an appropriate reference plasma sample volume. These solutions were stable for 7 days, at a temperature of 4 °C.

2.2. Chromatographic conditions

Chromatographic analyses were carried out on a liquid chromatography system (Shimadzu LC-20AT; Kyoto, Japan) equipped with a CBM-20A system controller and a fluorescence detector (Shimadzu RF-10 AXL). The interferon was analyzed in an RP 18 LichroCART® (125 mm × 4 mm × 5 μm particle size—Merck, Darmstadt, Germany) column, at room temperature (25 °C), with a mobile phase consisting of TFA solution 0.1% pH 2.5 and acetonitrile (86:14, v/v), using the isocratic mode at a flow-rate of 0.6 mL/min. The column effluent was monitored at λ_{ex} 295 nm and λ_{em} 335 nm. The mobile phase was filtered and degassed prior to use.

2.3. Plasma samples

Drug-free plasma samples from patients that had not been exposed to any drug for at least 72 h (blank plasma) were kindly supplied by Hospital das Clínicas de Ribeirão Preto, University of São Paulo, Brazil. These plasma samples were spiked with interferon alpha$_{2a}$ and used for optimization of the RAM in-tube SPME process and analytical validation of the developed method.

2.4. RAM (BSA-coated silica) capillary

For determination of the RAM capillary, silica particles (C18—45 μm) were slurried in methanol and packed into 50 mm (length) of polyether ether ketone (PEEK) tubing (1/16 in. O.D. and 0.02 in. I.D.). The capillary column was capped at both ends by a 1/16 in. (1 in. = 2.54 cm) zero-volume union fitted with a 10 μm frit. After this procedure, the capillary was conditioned with phosphate buffer (0.05 mol L^{-1}, pH 6.0) at a flow-rate of 1.0 mL min^{-1} for 20 min. The BSA immobilization was done in situ, based on the protocol proposed by Menezes and Felix [20], and by other authors [21–23]. Initially, 50 mL phosphate buffer solution (0.05 mol L^{-1}, pH 6.0) was percolated through the capillary at a flow rate of 1.0 mL min^{-1}; followed by 25 mL BSA solution 1.0 mg mL^{-1} (prepared in phosphate buffer solution), and by 25 mL glutaraldehyde solution (25%, v/v). After 5 h, the columns were washed with 10 mL sodium borohydride solution (1.0 mg mL^{-1}) and then with 60 mL water. The RAM–BSA column was stored in phosphate buffer solution (0.05 mol L^{-1}, pH 7.4) at 4 °C.

2.5. In-tube solid phase microextraction

The RAM capillary was fixed in the place of the injection loop of the LC system. The capillary connections were facilitated by placing MicroTight sleeves at each end of the capillary (Fig. 1).

In a glass vial (1.5 mL), 250 μL phosphate buffer (0.05 mol L^{-1}, pH 7.4) was added to 250 μL plasma sample spiked with interferon α_{2a}, resulting in a concentration of 3 MIU mL^{-1}. The sample was vortexed for 10 s before extraction. The extraction was carried out with the six port valve in the load position. Simultaneously, the analytical column was conditioned with the mobile phase. The plasma sample was directly injected into the RAM capillary. Extraction of each sample was possible by repeatedly aspirating (draw) and dispensing (eject) the sample through the capillary. After the draw/eject cycles, the capillary was washed with water (100 μL), to prevent contamination of the analytical column with residual endogenous plasma compounds. After the extraction, the valve was switched to the inject position, and the analyte was eluted (desorption process) from the RAM capillary with the mobile phase, and then transferred to the analytical column.

The in-tube SPME variables such as sample volume, draw/eject volume, number of extraction cycles (draw–eject), and desorption mode were optimized, to improve the sensitivity of the proposed method.

2.6. Analytical validation

The linearity was evaluated by calibration curves constructed using linear regression of the interferon peak areas (Y) versus the interferon nominal plasma concentration (X, MIU mL^{-1}). Accuracy and interday precision values were determined by means of quintuplicate in-tube SPME/LC assays of the blank plasma samples spiked

with interferon alpha solutions (quality control samples) representing the entire range of the calibration curve. Accuracy values were calculated by comparison of the concentrations of interferon α_{2a} added to the plasma samples with plasma interferon α_{2a} concentrations determined by the calibration curve. Furthermore, the selectivity of the method was investigated by comparison of the retention times of interferon α_{2a}, of other drugs, and of plasma endogenous compounds.

3. Results and discussion

The quantification of recombinant lower molecular weight proteins such as IFN-α_{2a} (~19 kDa) is often difficult, due to the nature of the drug, which contains host proteins, residual DNA, and other non-protein contaminants. Appropriate sample preparation is a critical step for the development of quantitative LC methods for determination of interferon α_{2a} in biological fluids [14]. Therefore, biocompatible in-tube SPME is a promising sample preparation approach for analyses of interferon α_{2a} in plasma samples.

The biocompatibility of the RAM material overcame the existing disadvantages of existing in-tube SPME phases, while the convenient format and minimal solvent requirements of SPME represented a significant improvement over the established RAM column switching approaches [24]. Moreover, the RAM–BSA (protein-coated silica) support has been widely employed, because of its low cost and its well established physicochemical properties, which has enabled its use in various chromatographic conditions, including the reverse elution mode [25].

3.1. In tube SPME conditions

The pH value has a significant effect on the extraction process based on the partition equilibrium, which should result in increased sensitivity, especially for basic and acid analytes [16]. Because of the isoelectric point of BSA (4.2 and 7.0) and interferon (5.9), the effects of the sample matrix and pH on the extraction of the studied compound were not evaluated. Therefore, the samples were diluted in phosphate buffer solution pH 7.4 0.05 mol L^{-1} [26]. The extreme pH values or higher buffer concentration could result in BSA and interferon denaturation, which would reduce the extraction efficiency.

Plasma sample volume (100, 250, 500, 600, and 700 μL) was optimized, to ensure adequate method sensitivity with minimum matrix effect. According to the obtained results (Fig. 2), there were no significant changes in sensitivity above 500 μL. However, 250 μL plasma sample was selected for subsequent analysis, to reduce the amount of plasma protein in the samples and thus save RAM capillary lifetime. The capillary protein exclusion capacity was also considered.

The draw/eject volume and the number of draw/eject cycles are related to the extracted amount, and also depend on the capacity of the column [27].

The sample draw/eject volume was evaluated from 50 to 500 μL. The extraction efficiency increased with larger volumes (Fig. 3). However, draw/eject volumes higher than 500 μL (one cycle) resulted in chromatograms with large baseline noise, indicating lower exclusion of the macromolecules. This could negatively affect the analytical precision of the method and the RAM–BSA column lifetime.

The number of the draw/eject cycles was varied from 1 to 4, to establish sorption equilibrium between interferon α and the RAM phase (Fig. 4). The extraction efficiency decreased with draw/eject cycles higher than one, probably because of partial drug desorption during each eject step.

Although the salting out effect (NaCl addition to the sample) can increase in-tube SPME efficiency, it should block the capillary. The salt solubility in water–organic mixtures is generally lower compared with that in pure water. Thus, the influence of salt addition to the sample matrix on the performance of in-tube SPME was not evaluated.

For the drug desorption, dynamic and static procedures were evaluated. Online elution was carried out by redirecting the mobile phase through the capillary (dynamic desorption). Desorption, or better, analyte elution with the mobile phase could generate peak broadening [16], which was not observed in this work. For the static desorption process, 50 μL mobile phase was aspirated and drawn to the capillary for static desorption for 2 min. After this proce-

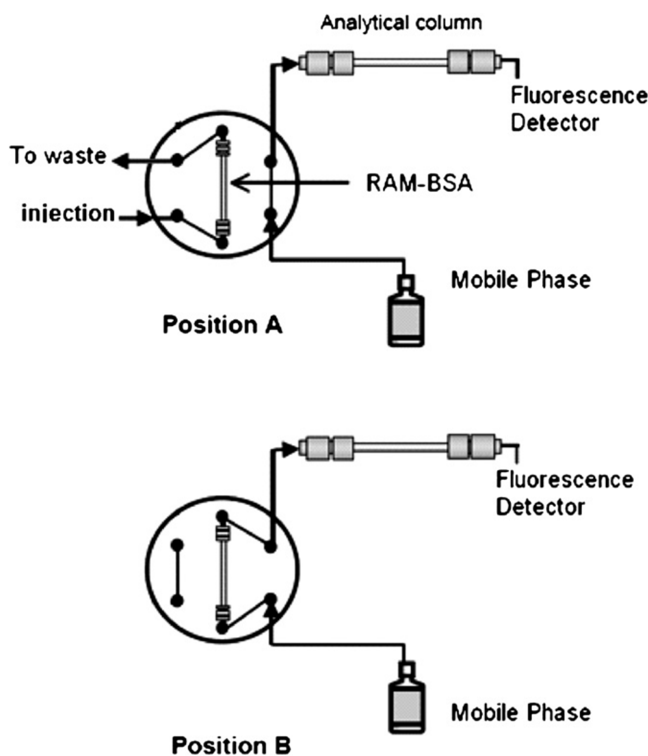

Fig. 1. Scheme of the operation mode of the six-port switching valve in the RAM in-tube SPME developed method.

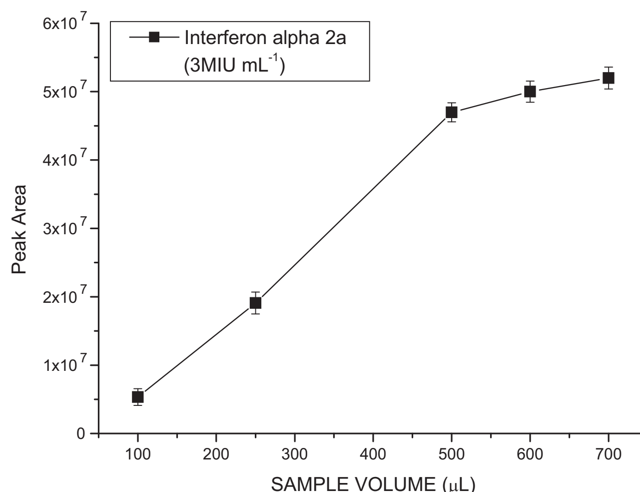

Fig. 2. Evaluation of the effect of the plasma sample volume on the performance of in-tube SPME/LC method. In-tube SPME conditions: plasma sample spiked with interferon α_{2a} (3 MIU mL^{-1}), diluted with phosphate buffer solution (0.05 mol L^{-1}, pH 7.4) at a 1:1, v/v ratio; draw/eject volume: 250 μL (1 cycle), desorption online with mobile phase.

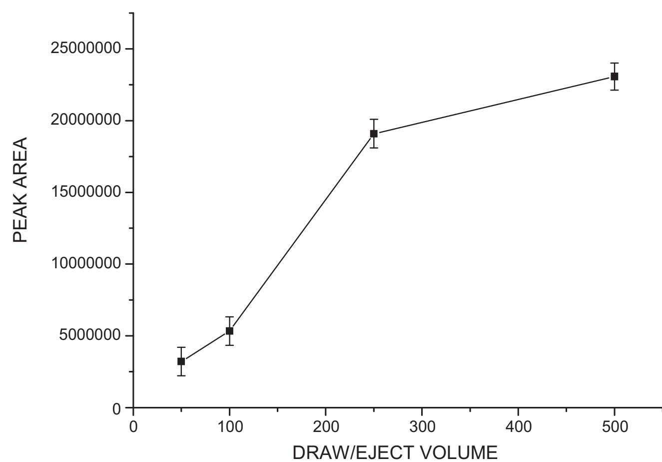

Fig. 3. Evaluation of the draw/eject volume on the performance of in-tube SPME/LC method. In-tube SPME conditions: plasma sample (250 μL) spiked with interferon α_{2a} (3 MIU mL^{-1}) diluted with of phosphate buffer solution (0.05 mol L^{-1}, pH 7.4) at a 1:1, v/v ratio; draw/eject cycle: 1, desorption online with mobile phase.

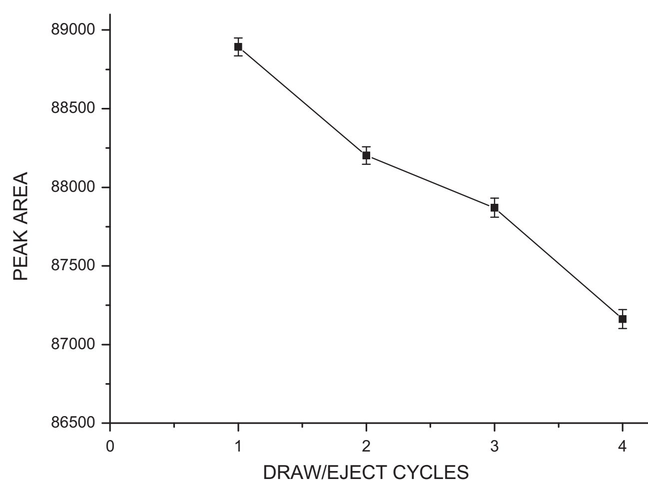

Fig. 4. Evaluation of the effect of the number of draw/eject cycles on the performance of in-tube SPME/LC method. In-tube SPME conditions: 250 μL of plasma sample spiked with interferon α_{2a} (3 MIU mL^{-1}), diluted with of 250 μL of phosphate buffer solution (0.05 mol L^{-1}, pH 7.4); draw/eject volume: 250 μL; desorption online with mobile phase.

dure, the six-port valve was switched to injection position, and the mobile phase transported the desorbed drugs to the LC column. The dynamic desorption procedure was selected for subsequent assays, to save analysis time. Moreover, the dynamic desorption procedure carryover did not observed.

After the chromatographic determination, the LC six-port valve was switched to the inject position, and the RAM–BSA capillary column was conditioned with water (100 μL) prior to the subsequent sample injection.

Thus, among the evaluated in-tube SPME/LC parameters, the best results were obtained with 250 μL spiked plasma sample diluted with 250 μL phosphate buffer solution (0.05 mol L^{-1}, pH 7.4), extraction in a single draw/eject cycle (250 μL), and online desorption (dynamic desorption) redirecting the mobile phase through the capillary.

3.2. RAM (BSA-coated silica) capillary column

The developed RAM (BSA-coated silica) capillary column consists of hydrophobic particles with the outer surface covered with bovine serum albumin (BSA). This makes the external surface of the particles compatible with a protein of the plasma sample that cannot penetrate into small pores. Plasma proteins were excluded by a chemical diffusion barrier created by a protein network at the outer surface of the particle. Hydrophobic groups (C18) at the inner surface are responsible for the interaction with the analyte. Interferon was extracted into the interior of the phase and enriched by partition [24,28].

RAM supports have been widely employed in the direct analysis of drugs in biological fluids, especially with the commercialization of the Biotrap support. This support presents an external surface modified by the plasma protein alpha 1-acid glycoprotein (AGP) in the Biotrap phase, which affords the required biocompatibility [29].

The RAM columns that use silica particles of 25 μm with C18 phase embedded within the porous center yield a molecular mass cutoff of 15 kDa [19,30]. In this work, C18 silica particles of the 45 μm were employed, which favored the pre-concentration of interferon α_{2a} (19 kDa) on the RAM–BSA capillary.

The most important step in the development of the RAM capillary column is the evaluation of the exclusion of plasma sample macromolecules. To this end, the chromatograms of the plasma sample spiked with interferon α_{2a} after RAM in-tube SPME/LC and conventional protein precipitation (saturated ammonium sulfate solution) analyses were compared, Fig. 5. Both analyses were carried out at the same interferon α_{2a} concentration (3.0 MIU mL^{-1}). Using both methods, some peaks in addition to that of the spiked analyte were observed. However, the RAM–BSA device provided a much cleaner extract from the biological fluid, as indicated by the reduced number and size of the peaks in the chromatographic baseline (Fig. 5b). The protein precipitated sample, on the other hand, extracted considerably more matrix components from the plasma matrix, thereby adding more potential interference. The RAM–BSA device also pre-concentrated the drug (C18), thereby increasing the analytical sensitivity of the proposed in-tube SPME/LC method.

The eluates from the RAM capillary column were collected, to analyze protein exclusion by the Bradford method [31]. To this end, the eluates of plasma samples that were injected into the RAM capillary (5 cm) were collected and diluted with Bradford reagent. After that, the absorbance (UV spectrophotometer, λ = 595 nm) of these eluates was compared with those values obtained for the same plasma samples percolated through the C18 capillary (5 cm).

According to the UV absorbance results at the maximum peak of the protein band, at a λ = 595 nm, the RAM–BSA capillary could exclude more than 90% of the plasma protein. The obtained results are in agreement with the exclusion profile found by Santos Neto et al. [22,23,32] and by Cass and co-workers [21,25] using conventional RAM–BSA columns.

The RAM capillary did not present clogging during the whole study. The developed RAM capillaries were reused more than 100 times without significant loss of the in-tube SPME efficiency. According to Neto et al. [32], it is important that the organic component methanol or acetonitrile of the mobile phase is not raised to a proportion higher than 15% until the macromolecules have been eluted.

The developed RAM–BSA capillary presented high backpressure. Therefore, increasing the acetonitrile volume in the mobile phase could reduce the viscosity and backpressure. Nevertheless, this practice could result in poor chromatographic resolution. Thus there should be a commitment between column backpressure, peak symmetry, adequate retention time, and resolution. The maximum acetonitrile concentration used in this work was 14%, as described in Section 2.1, to ensure higher method sensitivity.

No change in backpressure of the LC system was observed after several subsequent injections, which could prove the absence of proteins precipitation on the analytical column.

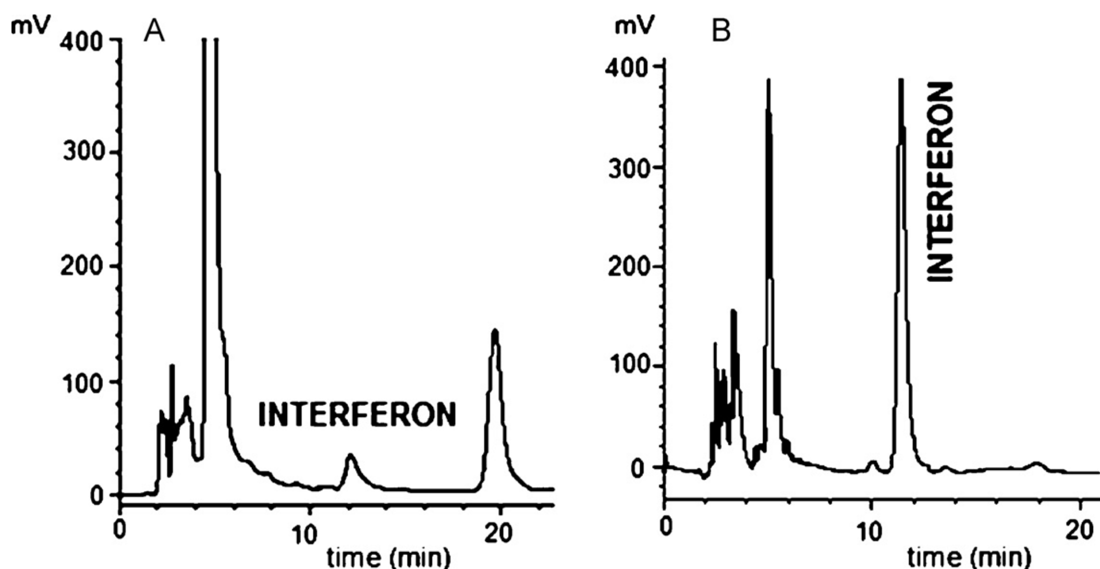

Fig. 5. Chromatograms of the plasma sample spiked with interferon α_{2a} (3.0 MIU mL^{-1}) after conventional protein precipitation (saturated ammonium sulfate solution, 1:1, v/v) (A) and RAM in-tube SPME/LC analysis (B).

3.3. Analytical validation of the RAM–BSA in tube SPME/LC method

The selectivity of the developed method is demonstrated by representative chromatograms of a drug-free human plasma sample (blank plasma) and drug-free human plasma sample spiked with interferon α at the therapeutic concentration (Fig. 6A and B).

The in-tube SPME/LC analysis of the blank plasma was carried out after analysis of the blank plasma spiked with interferon α_{2a} (3.0 MIU mL^{-1}). The absence of the analyte peak in the blank plasma chromatogram confirmed the complete elution of the extracted analyte from the previous sample, without carryover [24], Fig. 6B. The successful elimination of interference from endogenous compound in plasma samples could also be observed in the chromatogram of the blank plasma.

The linearity of the RAM–BSA in tube SPME/LC method was determined with plasma samples spiked with interferon (standard solution), which resulted in a concentration of 0.06 (LOQ), 0.25, 0.05, 1.0, 1.5, and 3.0 MIU mL^{-1}. The obtained regression equation and the corresponding correlation coefficient were $y = 80{,}246.2 + 256{,}699.9x$ and $r^2 = 0.998$, respectively. Each point of the calibration curve was performed in replicate ($n = 5$). Five different samples for each concentration were employed for this purpose. The LOQ value was determined as the lowest concentration in the calibration curve in which the CV was lower than 10%, based on a signal-to-noise ratio of about 10 (Table 1). The precision was determined by the percentage coefficient of variation (CV) (interassays) at three levels. The CV% values ranged from 3.1% to 7.9% (Table 1).

Table 1
Interday precision (coefficient of variation, CV) and accuracy of the RAM–BSA in tube SPME/LC method for interferon α_{2a} analysis.

Concentration (MIU mL^{-1})	Coefficient of variation (%)	Obtained concentration (MIU mL^{-1}) ± SD	Accuracy[a] (%)
0.06 (LOQ)	7.9	0.067 ± 0.004	114
0.75	6.3	0.80 ± 0.03	106
3.0	3.1	3.3 ± 0.09	110

[a] The accuracy was obtained in pentaplicate in the range of expected concentrations (3 levels × 5 replicates per level = 15 determinations).

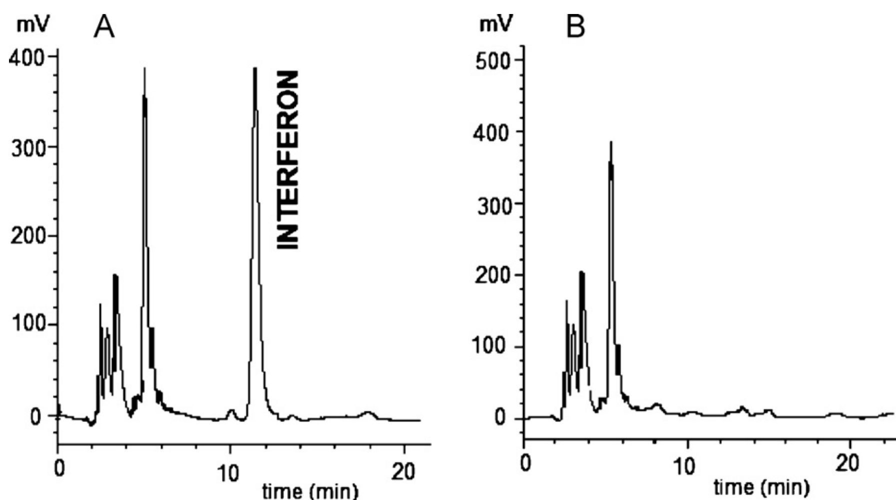

Fig. 6. RAM–BSA in tube SPME/LC chromatograms (A) drug-free plasma sample spiked with interferon α_{2a} at 3.0 MIU mL^{-1} and (B) drug-free plasma sample. The RAM–BSA in tube SPME/LC analyses were performed with 250 μL of plasma diluted with 250 μL of phosphate buffer 0.05 mol L^{-1} (pH 7.4), 1 draw–eject cycle (250 μL), online drug liquid desorption with mobile phase.

Table 2
Retention time of the drugs studied as possible interferents.

Drugs	Retention time (min)
Desipramine	2.18
Caffeine	2.25
Primidone	2.43
Citalopram	4.22
Clomipramine	4.56
Fluoxetine	5.63
Norfluoxetine	6.52
Sertraline	6.98
Interferon α_{2a}	11.88
Mirtazapine	12.91
Ribavarin	23.32
Amitriptiline	n.d.
Diazepam	n.d.
Propanolol	n.d.
Moclobemide	n.d.
Ascorbic acid	n.d.

n.d.—not detected.

The accuracy and interday precision of the RAM–BSA in tube SPME/LC method were assessed by replicate analysis ($n = 5$), using plasma samples spiked with interferon α_{2a} standard solution at different concentrations (Table 1).

The developed RAM–BSA support was able to exclude plasma proteins as well as pre-concentrate interferon α_{2a}, which resulted in high accuracy rates with lower coefficient of variation.

Yang et al. [10] obtained accuracy and precision within ±20% for interferon α_{2a} in human serum samples employing monolithic C18 solid phase extraction and LC–MS detection.

Interferon α_{2a} may be prescribed in combination with different psychotropic agents and other drugs, so it was important to assess the probable interference from potentially co-administered compounds (Table 2). On the basis of retention times, other drugs did not co-elute with the analytes.

The RAM–BSA in-tube SPME/LC method developed here presents adequate precision and accuracy for analysis of interferon α_{2a} for therapeutic drug monitoring. The analytical validation of the methodology showed that the method is highly selective for the analysis of interferon α_{2a} in plasma samples.

4. Conclusion

The present work demonstrates the appropriate application of the automated biocompatible in-tube solid-phase microextraction coupled with liquid chromatography (in-tube SPME/LC) for determination of interferon alpha$_{2a}$ (IFN α_{2a}) in plasma samples for therapeutic drug monitoring. The lab-made biocompatible (RAM, BSA-C18) capillary enables the direct injection of human plasma sample, as well as simultaneous macromolecule exclusion by chemical diffusion barrier and interferon α_{2a} pre-concentration.

The RAM–BSA in-tube SPME/LC method performed continuous extraction, concentration, desorption and injection online with a chromatographic system, which not only reduces the analysis time, but also provides adequate accuracy, precision, and sensitivity for determination of interferon α_{2a} in human plasma sample.

Thus, the proposed method can be a useful tool for the determination of interferon α_{2a} in plasma samples from patients receiving therapeutic dosages.

Acknowledgments

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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