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Monitoring bronchodilators with direct injection

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Abstract

A procedure was developed for the determination of caffeine and theophylline using a C_{18} column (5 μm, 250 mm × 4.6 mm) and micellar liquid chromatography using hybrid mobile phases containing sodium dodecyl sulfate (SDS) and propanol, butanol or pentanol as modifiers. Detection was performed with a variable wavelength UV–vis detector at 272 nm. After the application of an interpretative strategy for the selection of the optimimum mobile phase, caffeine and theophylline can be resolved and determined in serum samples by direct injection, using a mobile phase made up of 50 mM SDS–2.5% (v/v) propanol–10 mM KH_2PO_4, pH 7, with an analysis time below 5 min. Calibration was linear in the range 0.05 to 50 μg mL^{-1} with $r > 0.999$. The statistical evaluation of the method was examined by performing intra-day ($n = 6$) and inter-day calibration ($n = 7$) and was found to be satisfactory, with highly accurate and precise results. The proposed method was suitably validated and applied to the determination of caffeine and theophylline in serum samples of patients treated with bronchodilators.

Keywords: Monitoring of bronchodilators; Caffeine; Theophylline; Micellar liquid chromatography; Serum samples; Direct injection

1. Introduction

Methylxanthines are a class of drugs which affect pulmonary functioning. Caffeine (1,3,7-trimethylxanthine) and theophylline (1,3-dimethylxanthine) are ubiquitous in the human diet and are the most widely consumed psychoactive substances. Caffeine is an addictive drug and, among its various actions, it operates using the same mechanisms as those employed by amphetamines, cocaine and heroin to stimulate the brain. On a spectrum, the effects of caffeine are milder than those produced by amphetamines, cocaine and heroin but it manipulates the same channels and this is one of the reasons for the addictive qualities of caffeine. Theophylline stimulation of the central nervous system (CNS) is deeper and more dangerous than caffeine and appears on the list of forbidden substances in sports. Thus, the determination of methylxanthines is interesting in Monitoring in both the toxicological and the doping fields [1–8].

Numerous analytical methods have been reported for the analysis of caffeine and theophylline in biological fluids [9–36], including normal or RP-HPLC [9–23], GC [23] coupled with MS (GC–MS) [24,25], CE [26–28], immunoassay techniques [22,23,29–33], spectrophotometric techniques [34,35] or amperometric techniques [36]. A comparison of different analytical methods has also been made [22,23] but HPLC seems to be the most popular method for the determination of these compounds in serum samples using ultraviolet detection [9–13,16–23], phosphorimetric detection [14], MS [15] or modified column [9,16]. Gradient elution [18] and column switching methods [15] can resolve many interferences but, in general, they are more time-consuming than isocratic methods. However, the determination of caffeine and theophylline in biological fluids poses analytical problems, such as the presence of structurally similar metabolites and individual variability in the metabolic pattern, in conjunction with the fact that these compounds may only be present in low concentrations in the small sample that is difficult to extract from the matrices containing a variety of chemically different compounds [17]. On the other hand, chromatographic deter-

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mination requires a step involving HPLC extraction methods for caffeine and, in the case of theophylline, solid-phase extraction (SPE) [10,13,19,21] as well as liquid–liquid extraction (LLE) [11,17,18,20]. These extraction methods are tedious, time consuming, involve large sample volumes, consume important amounts of chemicals, including chlorinated solvents, which results in prohibitively expensive waste storage, segregation, disposal and environmental costs. Reduction of chemicals and solvents at the source would be an added advantage of the method. Moreover, for an extraction method to be deemed successful, both the removal of the analyte from the matrix and the trapping or concentration of the analyte, prior to analysis, must be optimized. In conclusion, the extraction procedures may be time-consuming, expensive and introduce additional sources of error.

Micellor liquid chromatography (MLC) is an alternative method to conventional liquid chromatography. The use of MLC for the separation of different samples is becoming more widespread because it offers a number of advantages with respect to HPLC. These include, for example, the low cost and toxicity of the mobile phases due to the small amount of solvent employed in the mobile phases, the enhanced selectivity and the simultaneous separation of hydrophobic and hydrophilic compounds. One of the main applications of MLC is the possibility of direct sample injection of biological material into the column because of the ability of micellar aggregates to dissolve sample proteins and other compounds [37]. This technique has proved to be a useful technique in the determination of diverse groups of drugs, including antihistamines, barbiturates, benzodiazepines, carbamates, diuretics, phenethylamines and corticosteroids in serum, urine and pharmaceutical samples [38–44]. In most cases, sodium dodecyl sulfate (SDS) has been used in a mixture with an organic modifier, i.e. propanol in the case of most hydrophilic solutes, and butanol or pentanol to decrease the retention times of most hydrophobic solutes.

The aim of the present paper was to develop a simple and sensitive MLC procedure for the screening and determination of caffeine and theophylline in serum with direct injection of the sample, which greatly simplifies the determination of these compounds. A mobile phase of SDS and a modifier with UV detection was used in the method described. This method can be useful for analyzing caffeine and theophylline in the field of Monitoring, and forensic and clinical toxicology.

2. Materials and methods

2.1. Standards and chemicals

Caffeine and theophylline were purchased from Sigma (St. Louis, MO, USA). Stock solutions of both compounds were prepared by dissolving the analyte in a few milliliters of methanol and later suitably diluted with water for analysis. The reagents used to prepare the micellar mobile phases were sodium dodecyl sulfate (99% purity, Merck, Darmstadt, Germany), methanol, 1-propanol, 1-butanol or 1-pentanol from Scharlab (Barcelona, Spain), disodium hydrogenphosphate and hydrochloride acid from Panreac (Barcelona, Spain). The standard solutions and the mobile phases were filtered through 0.45 μm nylon membranes of 12 and 45 mm diameter (Micron Separations, Westboro, MA, USA), respectively. Distilled-deionized water (Barnstead, Sybron, Boston, MA, USA) was used throughout.

2.2. Apparatus and instrumentation

Measurements of the pH were performed with a GLP 22 from Crison (Barcelona, Spain), equipped with a combined Ag/AgCl/glass electrode. The vortex shaker and sonification unit were from Selecta (Barcelona, Spain). The chromatograph, an Agilent Technologies Model 1100 (Palo Alto, CA, USA) was equipped with a quaternary pump, an autosampler with 2 mL vials fitted with a Rheodyne valve (Cotati, CA, USA) and a UV–vis detector. All the experiments were performed using a Kromasil C_{18} column with 5 μm particle size, 250 mm × 4.6 mm i.d. from Scharlab (Barcelona). The flow-rate was 1.0 mL min^{-1} and the injection volume 20 μL. UV detection was performed at 272 nm.

2.3. Sample preparation

Blood was collected in glass containers without any additives and was allowed to clot for 1 h at room temperature before the serum was separated by centrifugation at 3000 × *g* for 5 min at 5 °C. Within 8 h after collection, the serum was dispensed by 1 mL aliquots into sterile vials and used for analysis or frozen at −80 °C. Injection of the samples was performed within a maximum period of 24 h. The optimization of the procedure was performed with spiked serum samples containing accurately known amounts (25 μg mL^{-1}) of the compounds.

The high background signal due to the serum protein band can hamper the separation and detection of caffeine and theophylline by damaging the column material or by giving very poor reproducibility. Direct injection of spiked serum samples (without any dilution) mainly made the detection difficult as the protein band is wide and the fast eluting theophylline coelutes with the protein band. However, 1:10 dilution of the spiked serum samples with the micellar mobile phase used, before its injection, reduced the width of the protein band to allow the detection of the compounds. In these conditions, retention times remained unchanged at least after 500 injections into the chromatographic system. Before injection, serum samples were filtered directly into the autosampler vials through 0.45 μm nylon membranes.

2.4. Software used in data treatment

In the individual chromatograms obtained in these mobile phases, the retention factor (*k*), efficiency (*N*), asymmetry factor (*B*/*A*) and dead volume (determined as the mean value

of the first significant deviation from the base-line) were measured. A personal computer connected to the chromatograph through the HP Chemstation from Agilent (version A.09) was used for instrumental control, acquisition and treatment of chromatographic data. Michrom software was used for optimization studies [45] and Excel was employed in other calculations.

2.5. Recommended micellar liquid chromatographic method

The selected micellar mobile phase was 50 mM SDS–2.5% (v/v) propanol–10 mM KH_2PO_4, pH 7 (adjusted before the addition of the alcohol), with a flow rate of 1 mL min^{-1}, column thermostated at 25 °C, injection volume of 20 μL and a detection wavelength of 272 nm.

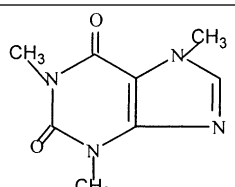
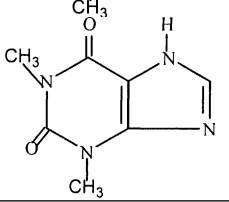
3. Results and discussion

3.1. Effect of pH and octanol–water partition coefficient

The protonation constants (Table 1) of caffeine and theophylline show two acid–base equilibria [46]. In SDS micellar media these values are displaced around 1–2 pH units. Furthermore, the working pH range of an unmodified C_{18} column is from 3 to 7. Thus, caffeine and theophyline are positively monocharged in the ranges 3–7 and 5–7, respectively. Experiments conducted using a pure micellar mobile phase of SDS 100 mM and hybrid mobile phases of SDS 100 mM–4% (v/v) propanol, SDS 100 mM–4% (v/v) butanol and SDS 100 mM–4% (v/v) pentanol buffered at pH 3, 5 and 7 indicated that the retention factors are not dependent on the pH. This behavior has been confirmed by comparing other mobile phases tested at different pH in the optimization step. So, pH 7 was selected as the optimum pH, as it is more adequate for the maintenance of the column.

The octanol–water partition coefficients, log $P_{o/w}$, shown in Table 1, indicated that the two analytes are hydrophilic substances. But the low value for theophylline (−0.02), in comparison with the value for caffeine (0.07), indicated that the elution order must be theophylline first and then caffeine. In all the mobile phases tested, albeit experimentally or through simulation, this order was maintained and no changes in selectivity were observed. Finally, the presence of the positive charge interacting with the negative charge of SDS allows these hydrophilic substances to be determined using MLC.

Table 1
Structures, pK_a and log $P_{o/w}$ values of caffeine and theophylline

Compound	Structure	pK_a	log $P_{o/w}$
Caffeine		0.6, 14	0.07
Theophylline		3.5, 8.6	−0.02

3.2. Effect of SDS concentration

In MLC efficiency is inversely proportional to the concentration of SDS and the retention factors decrease as concentrations of the surfactant rise. The same trend was followed by caffeine and theophylline. For example, in pure micellar mobile phases of SDS 50 mM, pH 7, the retention factor and efficiency values were 4.05, 900 and 2.55, 2000 for caffeine and theophylline, respectively, and using SDS 150 mM, pH 7, the values changed to 2.45, 650 and 1.60, 1700 for both substances. The retention factor decreases to 1.6 min and efficiency to/by around 300 U.

3.3. Effect of modifiers

In MLC, a small amount of short-chained alcohols is normally added as a modifier, in order to enhance efficiency and asymmetry as well as to decrease the retention factor and, therefore, the analysis time. This factor improves the overall chromatographic behavior of the individual compounds under study and allows the chromatographic behavior to be modulated. In the case of caffeine and theophylline, the effects of three organic modifiers, namely propanol, butanol and pentanol, were studied. Using 50 mM SDS, pH 7, if the concentration of propanol is 2.5% (v/v) the retention time and efficiency achieved were 2.46, 2100 for caffeine and 1.49, 3550 for theophylline (Fig. 1). When the concentration of propanol is increased from 2.5 to 12.5% (v/v), t_R and N decrease and increase, respectively, to 0.86, 4600 and 0.73, 5200 for both compounds (see Fig. 1). Using SDS 50 mM, pH 7, and 2% (v/v) pentanol, a long-chained modifier, t_R and N obtained were 0.72, 3230 and 0.6, 4200 for caffeine and theophylline, respectively, but on increasing the concentration of pentanol to 6% (v/v) these two parameters remain constant and the same behavior was observed using SDS 50 mM–butanol-1 and 7% (v/v) pH 7. Note that in butanol or pentanol, the retention factor is very small. The behavior described here was observed on checking all the other mobile phases containing SDS–propanol, or SDS–butanol and SDS–pentanol. Caffeine and theophylline are two hydrophilic substances that prefer to interact with the aqueous solvent of the mobile phase rather than with the micelles and if butanol or pentanol are used, due to their higher eluent strength, the two compounds appear near or in the dead volume. Only propanol offers the possibility of modeling the chromatographic behavior of the two compounds in order to obtain the optimum chromatogram in which res-

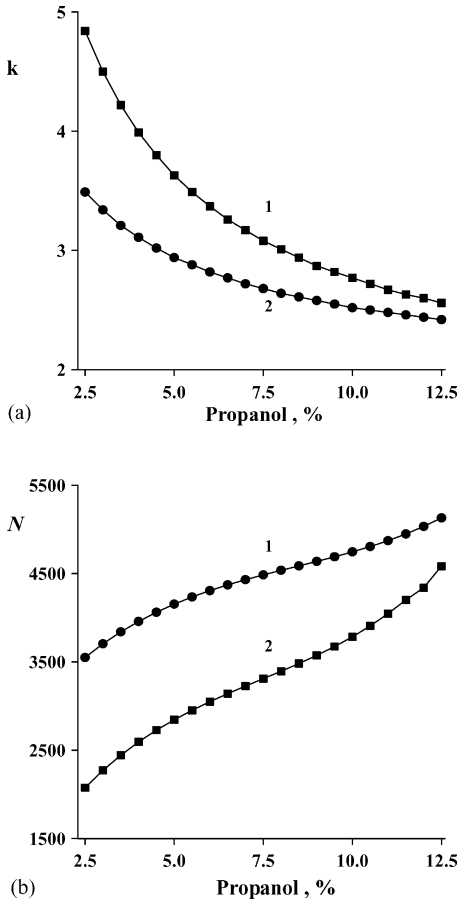


Fig. 1. Effect of propanol concentration on the retention factor (a) and efficiency (b) in the separation of the bronchodilators caffeine (1) and theophylline (2). Mobile phases were SDS 50 mM–% (v/v) propanol–10 mM KH_2PO_4 at pH 7.

olution and efficiencies are maximized and analysis time is minimized.

3.4. Optimization strategy and mathematical treatment

MLC has the capability to predict the retention of compounds using simple equations. The accurate prediction of the retention behavior, based on a checked model, can expedite the process of finding the optimal composition of the mobile phase for resolution and analysis of the two compounds under the criteria of minimum analysis time with an adequate resolution. The model employed for these predictions was as follows in Eq. (1) [45]:

$$k = \frac{K_{AS}(1 + K_{SD}\varphi)/(1 + K_{AD}\varphi)}{1 + K_{AM}[M](1 + K_{MD}\varphi)/(1 + K_{AD}\varphi)} \quad (1)$$

where $[M]$ and φ are the concentrations of surfactant and modifier, respectively; K_{AS} is the partition coefficient between stationary phase and water, K_{AM} is the binding constant between solute and micelle; K_{MD} and K_{AD} account for the displacement of the water–micelle equilibrium, whereas K_{SD} describe the modification of the water–stationary phase equilibrium. This equation was non-linearly fitted according to the Powell method [47] using the retention data from injections of the drug solutions in eight mobile phases containing SDS (mM) and propanol (%, v/v): 50–2.5, 150–2.5, 50–12.5, 150–12.5, 100–7.5, 50–7.5, 100–2.5 and 75–5, all of which contained 10 mM phosphate at pH 7. Serum blank, caffeine and theophylline were chromatographed in these mobile phases and the chromatographic parameters, i.e. retention factor, efficiency and asymmetry, were determined. It must be noted that, in the analysis of physiological fluids, the chromatographic data of the endogenous compounds and the protein band at the head of the chromatogram should be considered when selecting the mobile phase.

The accurate prediction of retention according to Eq. (1) allowed an interpretive procedure to be applied in order to predict the optimal resolution (r), following a criterion that utilizes the valley-to-peak ratios [48].

$$r = \prod_{i=1}^{n-1} \frac{X_{i,i+1}}{(\sum(x_{i,i+1}/(n-1)))^{n-1}} \quad (2)$$

where r is the global function of resolution, based on different properties, $X_{i,i+1}$, where $X_{i,i+1} = 1 - (h_1/h_2)$, being h_1 the height of the valley between two adjacent peaks, and h_2 the interpolated height between the maxima of both peaks measured at the abscissa of the valley. The global function of resolution, r, may vary from 0 to 1 and the proximity to one indicates the performance of the separation. The function was maximized to obtain the optimal mobile phase.

Incorporation of the shape of the chromatographic peaks in the optimization procedure improves the results. The reliable simulation of peak shape for any mobile phase of the variable space was carried out with an asymmetrical Gaussian function $h(t)$, where the standard deviation is a first-degree polynomial function [48]:

$$h(t) = H \exp\left[-\frac{1}{2}\left(\frac{t - t_R}{s_0 + s_1(t - t_R)}\right)^2\right] \quad (3)$$

where t is the time, H and t_R are the height and time at the peak maximum, respectively; s_0 is the standard deviation of the asymmetrical Gaussian peak describing the central region of the experimental peak; and s_1 a coefficient that quantifies its skewness. The coefficients s_0 and s_1 are related to the efficiency and asymmetry factor. These parameters were interpolated from the data obtained in the three experimental mobile phases closest to the simulated mobile phase. Using Eq. (1) and the mathematical treatment here described, the relative global error in the prediction of retention factors for the two drugs is below 1.6%. The coefficients in Eq. (1) for each drug enabled the mobile phase composition to be predicted for any desired retention time and provided a simple way to optimize the separation of mixtures.

3.5. Selection of optimum mobile phase

Using the optimization strategy described above, we have obtained the resolution diagram for SDS–propanol, pH 7, mobile phases. Complete resolution ($R \sim 1$) can be obtained with the exception of mobiles phases containing high amounts of SDS and propanol. In the low resolution mobile phase, theophylline elutes too fast and the result was that the protein band overlaps with the theophylline peak, at the head of the chromatogram. The mobile phase selected, under the criteria of maximum resolution–minimum analysis time–adequate chromatographic parameters, was SDS 50 mM–2.5% (v/v) propanol–10 mM KH_2PO_4, pH 7. In this mobile phase the retention time, efficiency and asymmetry factor were: 3.4–3600–1.2 for theophylline and 4.8–2100–1.4 for caffeine, respectively.

3.6. Selectivity

Under the conditions described above, the peaks for caffeine and theophylline (Fig. 2a) were symmetrical, well separated and free from interferences from the solvent front. The serum protein band elution finishes at 2.5 min and the first peak, corresponding to the theophylline, starts at 3.3 min. The blank chromatograms (Fig. 2b) do not show another endogenous substance peak. Other drugs often administered concurrently with caffeine or theophylline did not interfere with this assay, as was the case of ascorbic acid, codeine, ergotamine, guaifenesin, brompheniramine, butabarbital, chlorpheniramine, propyphenazone, papaverine, paracetamol, pantothenate, phenylephrine, prednisolone and steroids.

3.7. Linearity, sensitivity and limits of detection and quantification

Calibration curves were constructed for the individual compound, using the measured areas of the chromatographic peaks at six ($n = 6$) increasing concentrations that ranged from 0.05 to 50 $\mu g\,mL^{-1}$ in spiked serum samples. The slopes, intercepts and regression coefficients of the calibration curves are given in Table 2. The calibration curve was linear within the range of concentrations mentioned.

Table 2 also shows the limits of detection (LODs, *3s criterion*, $ng\,L^{-1}$) and limits of quantification (LOQs, *10s criterion*, $ng\,L^{-1}$). LODs were estimated as the concentration resulting in a signal-to-noise ratio of three (*3s criterion*) and, in the same way, LOQs using a ratio of 10 (*10s criterion*). The values of the LODs and LOQs of the MLC method were lower than those usually reported in the literature and allow the detection and quantification of the two bronchodilators, caffeine and theophylline, in serum with the method proposed in this work with direct injection of the sample and without any pretreatment.

Table 2
Calibration parameters, limits of detection ($ng\,mL^{-1}$, *criterion 3s*) and limits of quantification ($ng\,mL^{-1}$, *criterion 10s*) for caffeine and theophylline using the micellar mobile phase 50 mM SDS–2.5% (v/v) propanol, pH 7

Compound	Slope	Intercept	r	LOD	LOQ
Caffeine	40.1 ± 0.31	-1.19 ± 0.06	0.99996	3	30
Theophylline	46.6 ± 0.66	-5.86 ± 0.26	0.99998	3	25

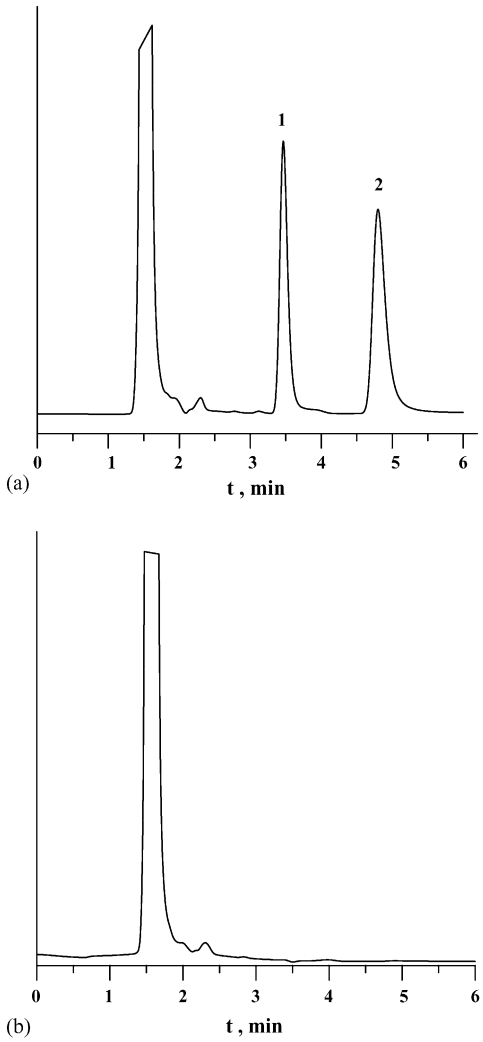


Fig. 2. Chromatogram of spiked serum samples (a) containing theophylline (1) and caffeine (2), and serum blank (b) obtained using the optimum mobile phase: SDS 50 mM–2.5% (v/v) propanol–10 mM KH_2PO_4 at pH 7.

3.8. Intra and interassay imprecision

Intra and interassay imprecision were determined for three different concentrations in the therapeutic range of caffeine (26–40 $\mu g\,mL^{-1}$) and theophylline (10–20 $\mu g\,mL^{-1}$). Intraassay imprecision values were calculated by measuring the areas of the peaks obtained by injection of series of 10 spiked serum samples at three different concentrations of caffeine and theophylline. The interassay imprecision was also obtained using the intraassay imprecision values gathered on 10 different days over a 3-month period. As shown in Table 3,

Table 3
Repeatability and intermediate precision at three different concentrations ($\mu g\,mL^{-1}$), c_1, c_2 and c_3 were 26, 33 and 40 and 10, 15 and 20, for caffeine and theophylline, respectively

Compound	R.S.D. (%, $n = 10$)					
	Repeatability			Intermediate precision		
	c_1	c_2	c_3	c_1	c_2	c_3
Caffeine	0.86	0.77	1.83	0.25	0.5	1.05
Theophylline	0.98	1.74	1.08	0.38	0.93	0.88

Table 4
Recovery assays for caffeine and theophylline in spiked serum samples

Compound	Added		Found, mean ± S.D. ($n = 5$)	
Caffeine	26	40	24.8 ± 0.3	39.8 ± 0.4
Theophylline	10	20	9.8 ± 0.4	19.7 ± 0.6

Concentrations are given in $\mu g\,mL^{-1}$.

overall mean imprecision, as defined by the percentage of relative standard deviation (R.S.D.), was near 1%.

3.9. Stability of the serum samples

The stability of the serum samples over a period of 3 months was assessed by preparing spiked serum samples containing 34 $\mu g\,mL^{-1}$ of caffeine and 15 $\mu g\,mL^{-1}$ of theophylline, divided into 15 aliquots in tubes protected from light and stored at −15 °C. Samples were removed at weekly periods and assayed to assess stability. The samples stored were stable over the 3-month period. Recoveries obtained ranged between 87 and 99%.

3.10. Application of the MLC method to the Monitoring of bronchodilators

In order to demonstrate the applicability of the assay, blank plasma samples, provided by the Analytical Service of the Verge dels Lliris Hospital in Alcoi (Alacant, Spain), were spiked with known amounts of the bronchodilators at two different concentrations within the therapeutic range for each substance. Table 4 shows the satisfactory recoveries data obtained. On the other hand, the MLC–UV method was used to analyze serum samples from patients ($n = 8$) treated with the bronchodilators caffeine or theophylline provided by the Hospital Verge dels Lliris d'Alcoi. These samples were analyzed and results compared with those obtained by the reference method used in the Hospital; good correlation was observed. The regression of the MLC versus HPLC methods gives slopes of 0.965 ± 0.033 and 0.982 ± 0.021, and y-intercepts of 0.012 ± 0.023 and 0.008 ± 0.015, for caffeine and theophylline, respectively.

4. Conclusions

This paper shows an example of the capability of MLC for the determination of monitorizable substances in serum samples. The bronchodilators caffeine and theophylline were determined directly by a simple, fast and reliable assay. In particular, the good separation of the two bronchodilators and the matrix band, achieved with the interpretive strategy of optimization, facilitates the selection of the optimum mobile phase, following the criteria of good resolution and minimum analysis time. Moreover, the direct injection of the sample complies with the above mentioned criterion of minimum analysis time. In other HPLC methods for the determination of the two bronchodilators, the separation is completed in times that are similar to those reported in this work, but they do not take into account the time required for the pretreatment of the sample, which includes extraction and preconcentration. In this way, the use of direct injection is clearly advantageous.

The selected mobile phase 50 mM SDS and 2.5% propanol, pH 7, uses small concentrations of reagents and these are nontoxic, compared with the normal solvents used in the RP-HPLC methods. In the MLC method, the analysis time is 5 min, intra- and inter-day precision is above 1.5, linearity is maintained within the therapeutic range of the two substances and, finally, LOQ and recoveries allow caffeine and theophylline to be determined at concentrations below the lower value of the therapeutic range and, for this reason, this assay can be used in the day-to-day analysis of bronchodilators in hospitals and for further studies with clinical and toxicological samples.

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References

[1] H. Hendeles, M. Weinberger, G. Johnson, Clin. Pharmacokinet. 3 (1978) 294.
[2] I. Hindmarch, P.T. Quinlan, K.L. Moore, C. Parkin, Psychopharmacology 139 (1998) 230.
[3] M.G. Myers, Ann. Int. Med. 114 (1991) 147.
[4] A.L. Bara, E.A. Barley, Cochrane Review, The Chochrane Library, Oxford, 2001, p. 2.
[5] J.D. Stookey, Eur. J. Epidemiol. 15 (1999) 181.
[6] T.W. Rall, Goodman and Gilman's The Pharmacological Basis of Therapeutics, eighth ed., McGraw-Hill, New York, 1993, p. 618.
[7] B.J. Lipworth, Lancet 350 (1997) 18.
[8] J.V. Aranda, D. Grondin, B.I. Sasyniuk, Ped. Clin. N. Am. 28 (1981) 113.
[9] P.J. Helmsing, R. Huisman, A. Van der Weele, Clin. Chem. 39 (1993) 1348.
[10] I. Papadoyannis, M. Georgarakis, V. Samanidou, G. Theodoridis, J. Liq. Chromatogr. 13 (1991) 1597.
[11] R. Ventura, C. Jimenez, N. Closaz, J. Segura, R. de la Torre, J. Chromatogr. B 795 (2003) 167.

[12] M.S. Bispo, M.C.C. Veloso, H.L.C. Pinheiro, R.F.S. de-Oliveira, J.O.N. Reis, J.B. Andrade, J. Chromatogr. Sci. 40 (2002) 45.
[13] M.S. Caubet, W. Elbast, M.C. Dubuc, J.L. Brazier, J. Pharm. Biomed. Anal. 27 (2002) 261.
[14] A.D. Campiglia, J.J. Laserna, A. Berthod, J.D. Winefordner, Anal. Chim. Acta 244 (1991) 215.
[15] H. Kanazawa, R. Atsumi, Y. Matsushima, J. Kizu, J. Chromatogr. A 870 (2000) 87.
[16] T. Umemura, R. Kitaguchi, K. Inagaki, H. Haraguchi, Analyst 123 (1998) 1767.
[17] M.B. Kester, C.L. Saccar, J. Chromatogr. 380 (1986) 99.
[18] T.E.B. Leakey, J. Chromatogr. 507 (1990) 199.
[19] K.A. Georga, V.F. Samanidou, I.N. Papadoyannis, J. Liq. Chromatogr. Rel. Technol. 23 (2000) 1523.
[20] G. Cheng, X.M. Wu, G. Lu, J.M. Chen, G.Q. An, X.G. Wang, D.H. Chen, L.P. Wang, Yaowu Fenxi Zazhi 17 (1997) 121.
[21] A. Schellen, B. Ooms, D. van de Lagemaat, R. Vreeken, W.D. van Dongen, J. Chromatogr. B 788 (2003) 251.
[22] Y.M. El Sayed, S.I. Islam, J. Clin. Pharm. Ther. 14 (1989) 127.
[23] L.M. Tsanaclis, J.F. Wilson, Ther. Drug. Monit. 19 (1997) 420.
[24] A. Takeda, H. Tanaka, T. Shinohara, I. Ohtake, J. Chromatogr. B 758 (2001) 235.
[25] K.A. Regal, W.N. Howald, R.M. Peter, C.A. Gartner, K.L. Kunze, S.D. Nelson, J. Chromatogr. B 708 (1998) 75.
[26] C.H. Feng, H.L. Wu, S.J. Lin, S.H. Chen, J. Liq. Chromatogr. Rel. Technol. 26 (2003) 1913.
[27] Y. Zhao, C.E. Lunte, J. Chromatogr. B 680 (1997) 265.
[28] W. Thormann, A. Minger, S. Molteni, J. Caslavska, P. Gebauer, J. Chromatogr. 593 (1992) 275.
[29] J.F.C. Wicks, A. Myring, J.F. Wilson, Ann. Clin. Biochem. 31 (1994) 291.
[30] H. Yuan, W.M. Mullet, J. Pawliszyn, Analyst 126 (2001) 1456.
[31] R.M. Garcinuno, P. Fernandez, C. Perez-Conde, A.M. Gutierrez, C. Camara, Talanta 52 (2000) 825.
[32] L. Tibi, D. Burnett, Ann. Clin. Biochem. 32 (1995) 339.
[33] L. Locascio-Brown, A.L. Plant, V. Horvath, R.A. Durst, Anal. Chem. 62 (1990) 2587.
[34] H.C. Goicoechia, A.C. Olivieri, A. Muñoz de la Peña, Anal. Chim. Acta 384 (1999) 95.
[35] K. Zhang, R. Cai, H. Huang, Z. Liu, Anal. Lett. 31 (1998) 1337.
[36] C.J. McNeil, J.M. Cooper, J.A. Spoors, Biosens. Bioelectron. 7 (1992) 375.
[37] A. Berthod, M.C. García-Alvarez-Coque, Micellar Liquid Chromatography, Marcel Dekker, New York, 2000.
[38] M. Gil Agustí, M.E. Capella Peiró, L. Monferrer Pons, M.C. García-Alvarez-Coque, J. Esteve Romero, Analyst 126 (2001) 457.
[39] M.E. Capella Peiro, D. Bose, A. Martinavarro Dominguez, M. Gil Agustí, J. Esteve-Romero, J. Liq. Chromatogr. Rel. Technol. 23 (2000) 1387.
[40] S. Carda Broch, J.S. Esteve Romero, M.C. García-Alvarez-Coque, Analyst 123 (1998) 301.
[41] M. Gil Agustí, J.R. Torres Lapasió, M.C. García-Alvarez-Coque, J. Esteve Romero, J. Chromatogr. A 866 (2000) 35.
[42] M.E. Capella Peiró, M. Gil Agustí, L. Monferrer Pons, J. Esteve Romero, Anal. Chim. Acta 454 (2002) 125.
[43] C. Quinones Torrelo, M. Martin Biosca, S. Sagrado, R.M. Villanueva Camañas, M.J. Medina Hernandez, Biomed. Chromatogr. 14 (2000) 287.
[44] Y. Martin Biosca, S. Sagrado, R.M. Villanueva-Camanas, M.J. Medina-Hernandez, Biomed. Chromatogr. 14 (2000) 113.
[45] J.R. Torres-Lapasió, A. Berthod, M.C. García Alvarez Coque (Eds.), Micellar Liquid Chromatography, Marcel Dekker, New York, 2000.
[46] The Merck Index, 13th ed., Merck and Co., Whitehouse Station, NJ, 2001.
[47] S.S. Rao, Optimization: Theory and Application, Wiley, New Delhi, 1985.
[48] M.C. García Alvarez Coque, J.R. Torres Lapasió, J.J. Baeza Baeza, J. Chromatogr. A 780 (1997) 129.