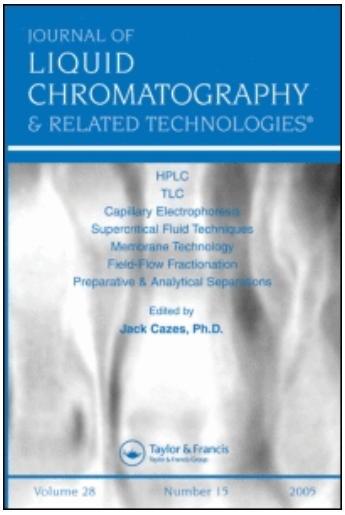

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Comparative Study of Solid-Phase Extraction Cartridges in the Simultaneous Reversed-Phase HPLC Analysis of Bamifylline and Its Major Metabolite AC-119 in Biological Fluids

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COMPARATIVE STUDY OF SOLID-PHASE EXTRACTION CARTRIDGES IN THE SIMULTANEOUS REVERSED-PHASE HPLC ANALYSIS OF BAMIFYLLINE AND ITS MAJOR METABOLITE AC-119 IN BIOLOGICAL FLUIDS

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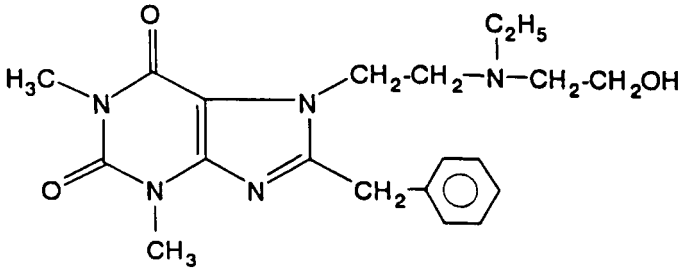
ABSTRACT

Several different solid-phase extraction cartridges provided by different manufacturers were investigated for the optimization of bamifylline and its major metabolite AC-119 isolation from samples of biological interest: human blood plasma and urine. The drug and its metabolite were subsequently analysed by HPLC after separation on a RP-18 Lichrosorb column 250x4.6 mm, 10μm, with caffeine as internal standard.

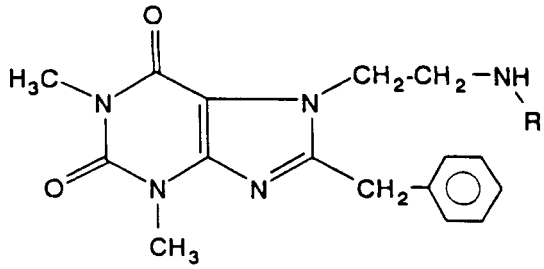
Total analysis time was approximately 10 min, while the sample volume required was low, 40 μl for blood plasma and 100 μl for urine samples. Data with respect to recovery, precision and accuracy and limits of detection are reported and discussed.

INTRODUCTION

Bamifylline (1), a xanthine derivative obtained by bisubstitution of theophylline acting as a bronchodilator is used in the therapy of asthma and



(1)



AC-119 R=-CH_2CH_3

AC-85 R=-CH_2CH_2OH

AC-115 R=-H

(2)

Fig. 1. Chemical structures of bamifylline (1) and its metabolite (2).

reversible airway obstructions. Its phamacokinetic and metabolic characteristics are significantly different than those of theophylline[1-2].

Three metabolites have been identified: AC-85, AC-155 and AC-119. From these, the latter was sufficiently separated, while the former two, only poorly[3].

Chemical structures of the compounds are shown in Fig. 1.

A very limited number of papers can be found in literature concerning bamifylline's and its metabolites' determination[4-7]. One of them is spectrophotometric, while the others are chromatographic with UV detection applied to pharmaceutical preparations and plasma from neonates or adults.

Sample volume required for plasma determinations is high, 1 ml, and total time of analysis quite long.

Bamifylline has been used as internal standard of HPLC analysis of mefenamic acid in pharmaceuticals and biological fluids[8], and in the analysis of tropane alkaloids, scopolamine and hyoscyamine, in feedstuffs and biological samples as well[9].

For the clean up procedure of biological samples the technique of solid-phase extraction was applied in order to isolate the compounds of interest from the sample matrix. This technique of extraction on off-line columns has become the most popular method for purification and isolation of substances present in biological materials e.g. urine, blood, tissues, etc[10].

The advantages of this technique, that transforms biological samples into analytical ones, include easy handling, saving in the extraction time in comparison to liquid-liquid extraction, a great variety of selective sorbents provided by several manufacturers and high recovery percentages as reported for a wide range of compounds[11]. These recovery rates are influenced by many factors, consequently is the reproducibility of the analysis as well. Among these factors, differences in packing material from batch to batch or from one manufacturer to another should not be ignored, as being connected to interactions between surface-matrix, isolated substance-mobile phase and/or irreversible sorption which can take place during the performance of the process. In this connection the choise of the packing material with proper selectivity may be very important, especially when biological samples are to be treated by solid-phase extraction in order to isolate compounds of interest from endogenous interferences[12-15].

In the present paper, a modification from a previously developed method by the authors is proposed[16]. This modification is regarding to the flow rate of the eluent required for improving the separation of components of interest from interfering compounds existing in biological matrices.

Concerning solid-phase extraction of bamifylline and AC-119 from biological matrices, different sorbents from different manufacturers were

compared on the basis of the drug relative recoveries using caffeine as internal standard.

EXPERIMENTAL

Chemicals and Reagents

Bamifylline and AC-119 metabolite were kindly provided by ALFA WASSERMANN, SpA Bologna, Italy, and were used without further purification.

Caffeine used as internal standard, was purchased by Sigma (St. Louis, MO, USA).

HPLC gradient grade methanol and acetonitrile were obtained from MERCK (Darmstadt, Germany).

Ammonium acetate pro analysi was also from MERCK. All other reagents used were of analytical grade. Bis de-ionised water was used throughout analyses. Pharmaceutical preparations with bamifylline as active ingredient, under the trade name Trentadil, were purchased by Rhône-Poulenc, France.

Bond Elut cartridges for SPE were provided by Analytichem, a division of Varian (Harbor City, U.S.A.).

Bakerbond cartridges were gratis provided by J.T. Baker (Gross Gerau, Germany).

Alltech cartridges were supplied from Alltech (Deerfield, IL, U.S.A.).

Separcol minicolumns were donated by Polymer Institute, Slovak Academy of Sciences, (Bratislava, Slovakia), and Anapron spol sr.o (Bratislava, Slovakia).

Apparatus

The chromatographic system which was operated in isocratic mode, used for the simultaneous analysis of bamifylline and AC-119 metabolite, consisted of the commercial components: an SSI 222D Pump (State College,

PA, U.S.A.), an SSI 500 variable UV/VIS Detector operated at 277 nm and a sensitivity setting of 0.002 absorbance units full scale (AUFS), a 9125 Rheodyne (California, U.S.A.) injection valve with a 20 μl loop and a HP 3396 II integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

The analytical column Lichrosorb RP-18 250x4.6 mm, 10 μm, was purchased from Alltech (Deerfield, IL, U.S.A.).

The solid - phase extraction procedure for the biological samples' pretreatment was performed on a Vac Elut vacuum manifold column processor purchased from Analytichem International, a division of Varian (Harbor City, U.S.A.). All evaporations were performed with a 9-port Reacti-Vap evaporator (Pierce, Rockford, IL, U.S.A.).

UV spectra for selecting the working wavelength of detection were taken using a Varian DMS 100 S UV/VIS double-beam spectrophotometer. All computations were achieved using a VIP 312 Computer.

Chromatographic conditions

A reversed phase C18 Lichrosorb column 250x4.6 mm, 10 μm, was used for separation of analytes, at ambient temperature 22°C. The mobile phase consisted of CH_3OH-0.05 M CH_3COONH_4 (67:33 v/v) at a flow rate of 1.5 ml/min, was selected among others investigated, for leading to optimal resolution of compounds, as well as to convenience regarding total time of analysis. Results were satisfactory for standards and pharmaceutical preparations but not for biological samples[16].

At this flow rate compounds of interest were not sufficiently separated from interfering compounds existing in sample matrices. This reason urged to a modification of this parameter when the method was applied to samples of biological origin. Thus the flow rate was reduced to 0.75 ml/min.

Prior to use mobile phase was filtered through 0.2 μm membrane filters Anodisc 47 (Alltech, Deerfield, IL, U.S.A.), and degassed by sonication in an ultrasonic bath.

Caffeine was chosen to be used as internal standard after an assay of a wide variety of organic compounds, taking into consideration the sufficient resolution, as well as the spectral criteria.

System suitability

The separation between compounds as shown in chromatograms presented in Fig. 2 and Fig. 3, is complete as signified from resolution factors R_t as well. These were found to be 2.78 and 4.10 in urine samples and 2.11 and 3.41 in plasma samples between caffeine and AC-119 and between caffeine and bamifylline respectively.

Selectivity and Extraction efficiency

The selectivity of the developed RP-HPLC method for the simultaneous determination of bamifylline and its metabolite AC-119 in presence of caffeine, internal standard, was investigated at their retention times.

Solid phase extraction was found to be appropriate for biological samples' purification as soon as no interference from endogenous, compounds from sample matrices were observed in their chromatograms (Fig. 2 and Fig. 3).

Therefore the proposed method can be applied to the analysis of bamifylline and AC-119 in presence of caffeine, in biological samples.

Extraction efficiency was calculated by extracting standard solution containing 5.0 ng/μl of bamifylline and 5.85 ng/μl of AC-119 and 0.784 ng/μl of caffeine, internal standard. This sample was extracted according to the procedure described under solid - phase extraction procedure. Recovery of compounds was calculated by comparing the peak area ratios against internal standard with those obtained for unextracted methanolic solutions containing similar concentrations of bamifylline and AC-119.

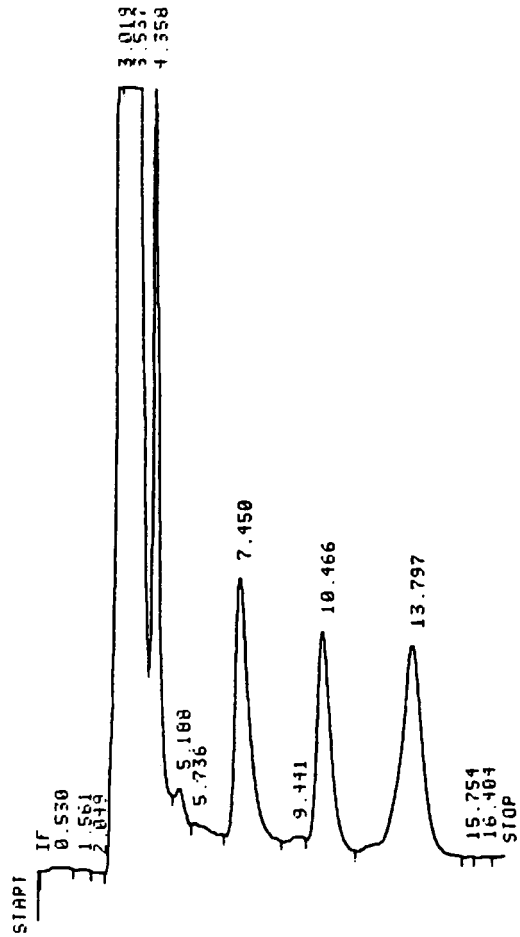


Fig. 2. High-performance liquid chromatogram of Bamifylline and AC-119 metabolite extracted by Solid-Phase Cartridges C8, from Human Blood Plasma.
Peaks: (4.358 min) Caffeine, 0.784 ng/µl. (7.450 min) AC-119, (10.466 min) Bamifylline at 2.0 ng/µl, (13.797 min) unknown.
Chromatographic conditions are given in text.

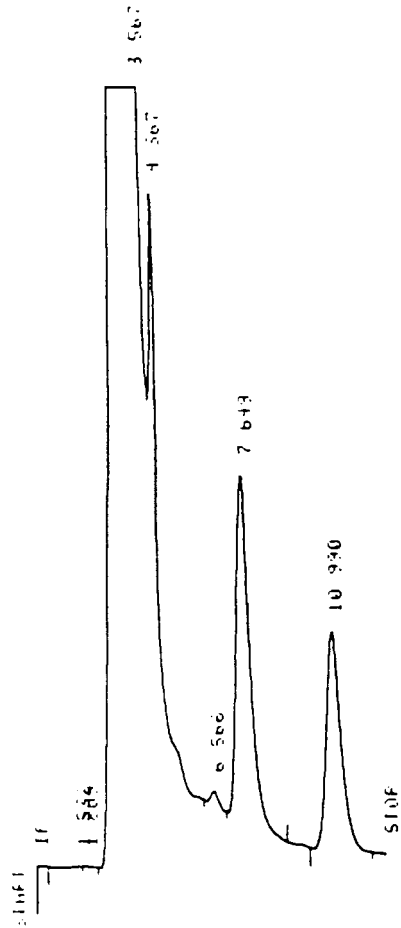


Fig. 3. High-performance liquid chromatogram of Bamifylline and AC-119 metabolite extracted by Solid-Phase cartridges C18 from Urine. Chromatographic conditions are given in text. Peaks: (4.367 min) Caffeine 0.784 ng/μl. (7.649 min) AC-119 and (10.990 min) Bamifylline at 3.0 ng/μl.

Linearity of calibration curve - Limits of detection and quantitation

Linearity was observed up to 15.0 ng/µl and 20.0 ng/µl for AC-119 and bamifylline respectively. The limits of detection were assessed in the presence of internal standard caffeine. Those were considered to be the quantities producing a signal of peak height twice the size of background noise, and found to be 1.0 and 2.0 ng for AC-119 and bamifylline respectively.

Limits of quantitation were calculated on the basis of regression equation data and found to be 1.352 ng/µl and 0.531 ng/µl for AC-119 and bamifylline respectively, in blood plasma samples and 0.651 ng/µl and 0.919 ng/µl for AC-119 and bamifylline respectively in urine samples.

Preparation of standard solutions - Calibration curve

Stock standard solutions of 100 ng/µl in bamifylline, AC-119 and caffeine were prepared in methanol and stored at 4°C. Working standard solutions were prepared by appropriate dilution with MeOH.

Calibration curve was constructed in the presence of 0.784 ng/µl of internal standard at concentrations of 0.50 - 2.0 - 3.0 - 5.0 and 7.0 ng/µl for bamifylline and metabolite AC-119, using spiked samples of biological fluids.

Aliquots of 20 µl of these solutions were injected into the HPLC system and peak area ratios of bamifylline and AC-119 to those of caffeine were recorded and plotted versus bamifylline and AC-119 concentrations.

All determinations were repeated eight times and results were treated statistically.

Biological Sample Preparation

a. Human blood plasma

Aliquots of 40 µl of drug-free human blood plasma were treated with 80 µl of CH_3CN in order to precipitate proteins. After vortex mixing for 2 min the sample was spiked with 100 µl of bamifylline - AC-119 standard solutions at concentration levels of 0.50 - 2.0 - 3.0 - 5.0 and 7.0 ng/µl containing 0.784 ng/µl caffeine internal standard.

Then the sample was centrifuged at 3500 g for 15 min and the supernatant was evaporated at 45°C under nitrogen stream to remove organic solvents. Subsequently the sample was slowly applied to the solid-phase cartridge.

b. Urine

The same sample preparation method was followed for urine samples after a small modification regarding sample volume. Thus in 100 μl of urine sample, 200 μl of acetonitrile were added and the procedure was followed as described above.

Solid - Phase Extraction Procedure

Solid - phase cartridges, fitted to the vacuum manifold, were conditioned by flushing with 3 ml methanol and 3 ml of water prior to the addition of the sample. After passage of the sample containing the internal standard through the cartridge, the sorbent was washed by 3 ml of CH_3COONH_4 0.05 M and 3 ml of water. Then the cartridge was dried by sucking air and the elution of the retained drugs was performed by applying 3 ml of methanol. The eluates were collected into 3 ml cone vials. The solvent was subsequently evaporated to dryness at 45°C under stream of nitrogen. The dry residue was redissolved in 100 μl of MeOH by agitating on a Vortex for 10 sec.

Aliquots of 20 μl of this solution were injected onto the analytical column of HPLC.

RESULTS AND DISCUSSION

There are not many literature references concerning determination of bamifylline and AC-119, its major metabolite. In the existing literature, determination methods proposed are time consuming and require significantly large volumes of biological samples. Solid-phase extraction procedure used for clean up and preconcentration of compounds of interest proved to be selective and thus suitable for application to biological sample pretreatment.

Table 1. Statistical Evaluation of Analysis Data

Blood Plasma Bamifylline	$Y=(-1.7564 \cdot 10^{-2} \pm 0.125226) + (0.269185 \pm 0{,}040104)X$
Solutions	$r=0.9999$ LOQ=0.531 ng/μl
Blood Plasma AC-119	$Y=(-5.2899 \cdot 10^{-2} \pm 0.419244) + (0.428148 \pm 0.115876)X$
Solutions	$r=0.9961$ LOQ=1.352 ng/μl
Urine Bamifylline	$Y=(1.0587 \cdot 10^{-2} \pm 0.098805) + (0.153759 \pm 0.025046)X$
Solutions	$r=0.9986$ LOQ=0.919 ng/μl
Urine AC-119	$Y=(0.101519 \pm 0.066268) + (0.163605 \pm 0.014357)X$
Solutions	$r=0.9992$ LOQ=0.651 ng/μl

X= Concentration of bamifylline or AC-119 in ng/μl.

Y= Peak area ratio of bamifylline to caffeine.

Regression equation is calculated as:

$$Y=(a \pm t_{(0.05,\, n-2)}\, Sa) + (b \pm t_{(0.05,\, n-2)}\, Sb)\, X$$

LOQ = Limit of Quantitation = $\frac{t \cdot Sa}{b} + Sx_i$ where

$$Sx_i = \frac{So}{b} \sqrt{1 + \frac{1}{n} + \frac{(y_i - \bar{y})^2}{b^2\,(\Sigma x^2 - n\bar{x}^2)}} \quad \text{in ng/μl (17).}$$

r = Correlation coefficient.

Table 2. Solid-phase extraction cartridges from different manufacturers investigated in the present study for Bamifylline and AC-119 recovery

Cartridge	Recovery[a] (%)		Notes
	Bamifylline	AC 119	
Separcol C1, 200 mg/3ml	140.15 ± 13.12	125.13 ± 14.18	1
Separcol C8, 200 mg/3ml	121.08 ± 14.19	141.38 ± 35.46	1
Separcol C18, 200 mg/3ml	133.34 ± 19.28	141.54 ± 23.24	1
Bondelut C8, 200 mg/3ml	89.53 ± 14.42	101.62 ± 21.90	2
Bondelut C18, 200 mg/3ml	86.78 ± 3.86	73.86 ± 1.98	
Alltech C18, 200mg/3ml	86.39 ± 5.12	80.82 ± 3.18	
Alltech C18, 100mg/3ml	104.74 ± 2.33	102.3 ± 4.15	2
Bakerbond C18, 200mg/3ml	100.26 ± 3.66	92.82 ± 3.53	2
Bakerbond C18, 500mg/3ml	123.18 ± 24.86	141.2 ± 31.98	1
Separcol S1 C18 L,200 mg/3ml			3
Separcol C18 RPS,200 mg/3ml	52.52 ± 7.63	43.36 ± 5.71	
Separcol C18 M, 200 mg/3ml	> 200	> 200	1
Separcol C18 T, 200 mg/3ml	22.65 ± 2.43	12.35 ± 3.36	
Bakerbond Narc-1, 250 mg/3ml	56.43 ± 10.53	13.67 ± 6.74	
Bakerbond Narc-2, 125 mg/3ml	30.45 ± 5.18	-	4

a Mean value from three cartridges.

1. Low recovery of caffeine yields over 100% recovery for the other compounds.
2. Selected for further investigation.
3. Almost none caffeine recovery, thus no calculation of bamifylline and AC-119 recovery can be performed.
4. No peak found for AC-119.

Table 3. Bamifylline and AC-119 recoveries from blood plasma and urine samples after SPE

Type of Cartridge		Recovery (%)			
		Bamifylline[a]	RSD (%)	AC-119	RSD (%)
Plasma					
	Bondelut C8, 200 mg/3ml	85.33 ± 6.62	7.75	84.60 ± 6.21	7.34
	Bakerbond C18[c], 200 mg/3ml	89.39 ± 2.94	3.28	79.53 ± 2.40	3.01
	Alltech C18[b], 100 mg/3ml	80.06 ± 6.64	8.29	66.32 ± 2.01	3.02
Urine					
	Alltech C18, 100 mg/3ml	126.65 ± 1.27	1.00	117.56 ± 0.13	0.11
	Bakerbond C18, 200 mg/3ml	166.78 ± 6.05	3.62	157.39 ± 4.68	2.97
	Bondelut C8, 200 mg/3ml	146.27 ± 10.20	6.97	163.74 ± 11.23	6.85

a. Mean value from three cartridges ± SD.

b. Recovery seems adequate but this only due to low recovery of caffeine.

c. This type could be equally used, but not enough cartridges were available for further applications.

Total chromatographic analysis time was approximately 10 min.

Table 1 shows the results of statistical evaluation of analysis data.

For the selection of the cartridge with best extraction efficiency, several sorbents from different manufactures were investigated for bamifylline and AC-119 recovery from methanolic solutions at a concentration of 5.0 and 5.85 ng/μl respectively, with caffeine as internal standard 0.784 ng/μl. Recovery results are shown in Table 2.

Table 4. Recovery of Bamifylline and AC-119 from human blood plasma after SPE on C8 cartridges using Caffeine as internal standard

Compound	Added (ng)	Found[a] ± SD (ng)	RSD (%)	Recovery (%)
Bamifylline	40.0	40.54 ± 2.26	5.58	101.35
	60.0	58.42 ± 3.04	5.20	97.37
	100.0	73.76 ± 2.14	2.90	73.76
AC-119	46.8	40.31 ± 3.50	8.67	86.13
	70.2	69.17 ± 4.02	5.81	98.53
	117.0	83.56 ± 2.64	3.16	71.42

a Mean value of five measurements ± SD

Three types of SPE cartridges, as shown in Table 2, were found to be the most suitable for the recovery of bamifylline and AC-119 from methanolic solutions. Therefore they were chosen among the others for further investigation with blood plasma and urine as sample matrices spiked with 5.0 and 5.85 ng/μl of bamifylline and AC-119 respectively with 0.784 ng/μl caffeine, the internal standard.

Results are presented in Table 3.

From the results obtained for the recoveries from the three different types of cartridges, presented in Table 3, the following cartridges were selected: (1) Bondelut C8 for extraction of bamifylline and AC-119 from blood plasma, although Bakerbond C18 could be equally used, but there were not enough cartridges available, thus the former were preferred over the latter.

(2) Alltech C18 100 mg/3ml cartridges were selected for extraction of the compounds of interest from urine matrix, though the other two types gave higher recovery due to insufficient extraction of caffeine.

Table 5. Recovery of Bamifylline and its metabolite AC-119 from human urine samples after SPE on C18 cartridges with Caffeine as internal standard

Compound	Added (ng)	Found[a] ± SD (ng)	RSD (%)	Recovery (%)
Bamifylline	60.0	57.35 ± 2.27	3.96	95.58
	100.0	71.82 ± 3.08	4.29	71.82
	140.0	123.09 ± 2.02	1.64	87.92
AC-119	70.2	68.64 ±3.48	5.08	97.78
	117.0	88.46 ± 2.13	2.41	75.61
	163.8	143.29 ± 10.60	7.39	87.48

a Mean value of five measurements ± SD

Table 6. Reproducibility of Bamifylline and AC-119 recovery from blood plasma after extraction on four different cartridges of same manufacturer

Compound	Cartridge	Added (ng)	Found[a] (ng)	RSD[b] (%)	Recovery (%)
Bamifylline	1	40.0	42.23 ± 4.61	2.88	105.57
	2		44.68 ± 3.28		111.7
	3		43.94 ± 3.56		109.85
	4		42.53 ± 4.18		106.32
AC-119	1	46.8	47.18 ± 6.75	12.68	100.81
	2		53.47 ± 5.24		114.25
	3		48.04 ± 6.29		102.65
	4		45.66 ± 4.37		97.56

a Mean value of six determinations ± SD

b Overall RSD, n = 24

Table 7. Reproducibility of Bamifylline and ac-119 recovery from urine after SPE on four different cartridges from the same manufacturer with the same sorbent

Compound	Cartridge	Added (ng)	Found[a] (ng)	RSD[b] (%)	Recovery (%)
Bamifylline	1	60.0	41.70 ± 6.13	7.03	69.50
	2		68.16 ± 7.81		113.60
	3		58.04 ± 10.21		96.73
	4		46.78 ± .0.91		77.98
	1	140.0	117.76 ± 8.45	6.23	84.11
	2		120.42 ± 10.34		86.01
	3		119.26 ± 2.30		85.18
	4		129.02 ± 8.98		92.16
AC-119	1	70.2	62.26 ± 8.54	20.81	88.69
	2		100.97 ± 7.38		143.83
	3		78.37 ± 7.56		111.64
	4		71.06 ± 1.31		101.23
	1	163.8	116.31 ± 10.37	6.05	71.01
	2		127.29 ± 10.75		77.71
	3		130.77 ± 3.17		79.84
	4		146.46 ± 8.34		89.41

a Mean value of six determinations ± SD

b Overall RSD, n = 24

As mentioned before, caffeine was added in each sample at a concentration level of 0.784 ng/μl.

The reproducibility and accuracy of solid-phase extraction of bamifylline and AC-119 from biological samples were investigated by spiking drug free blood plasma and urine with known concentrations of the compounds and then by comparing the peak area ratios against internal standard with those obtained when extracting methanolic standard solutions containing comparable concentrations of compounds.

Results are given in Table 4 and Table 5. Each value represents the mean of five measurements carried out.

Tables VI and VII show the recovery of bamifylline and AC-119 from blood plasma and urine respectively after solid-phase extraction on four different cartridges from the same manufacturer with the same sorbent, in order to check out the reproducibility of extraction.

The mean conclusion which can be drawn from the point of view of practical utilization of the results presented, is that a sorbent can not be defined as good or bad. A sorbent which manifests good properties towards one drug can be unfit in extraction of another and vice versa.

For this reason a sorbent that shows higher recoveries for the majority of compounds of interest is to be selected for showing optimal behaviour for the analysis.

Recoveries of the drugs from methanolic solutions are significantly higher than those from biological samples. This can be explained by the fact that endogenous compounds from sample matrix occupy active sites of the sorbent surface and as a consequence the possibility to form interactions between sorbent and drugs is reduced.

Manufacturer - to - manufacturer variations are due to the fact that it is not yet achievable to unify the properties of silica and bonding procedures. The only solution to this problem seems to be the use of cartridges of same manufacturer.

CONCLUSION

The HPLC analysis method of bamifylline and its major matabolite AC-119 described at the present study is characterised by high accuracy, precision and reproducibility, high sensitivity and satisfactory selectivity. Solid-phase extraction procedure has good recovery efficiency and sufficient removal of endogenous compounds.

Volumes of biological fluids required are small and separation time, approximately 10 min, which renders the technique applicable to clinical analysis of bamifylline and its metabolite AC-119.

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