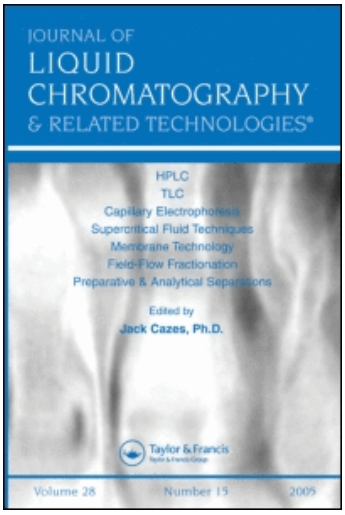

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SIMULTANEOUS DETERMINATION OF BAMIFYLLINE AND ITS MAJOR METABOLITE AC-119 BY RP-HPLC

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ABSTRACT

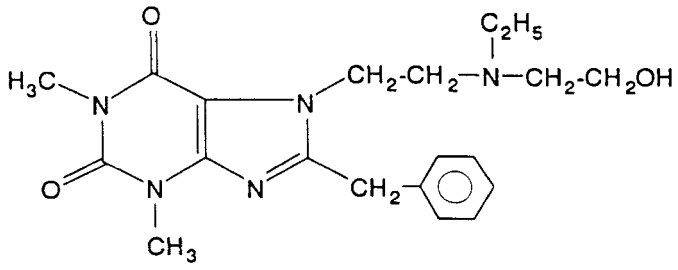
In the present study a simple, sensitive accurate and fast method for the simultaneous determination of bamifylline and its major metabolite AC-119 by isocratic reversed-phase HPLC is developed. Caffeine is used as internal standard for quantitation analysis and the method was applied to the determination of bamifylline in pharmaceutical preparations (tablets and suppositories).

The isocratic elution was performed with methanol - 0.05 M ammonium acetate (67:33 v/v) at a flow rate of 1.5 ml/min, using a Lichrosorb C_{18} analytical column 250x4.6 mm, 10 μm. Absorbance was monitored at 277 nm. Total analysis time was approximately 5 min.

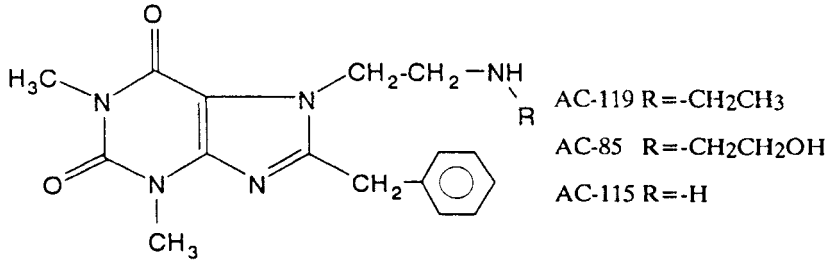
Data with respect to precision and accuracy, and limits of detection are reported and discussed. The described method can be readily utilised for pharmaceutical products analysis and pharmacokinetic studies as well.

INTRODUCTION

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



(1)



(2)

Fig. 1. Chemical structures of bamifylline (1) and its metabolites (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].

In the present paper a simple, accurate and rapid isocratic HPLC method for the simultaneous determination of bamifylline and its major metabolite AC-119 using caffeine as internal standard is developed. The method appears to be suitable for therapeutic determinations and pharmacokinetic investigations as well, due to its sensitivity, low limits of detection, selectivity and lack of interferences.

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.

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 a Lichrosorb RP-18 250x4.6 mm, 10 μm, was purchased from Alltech (Deerfield, 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 C_{18} 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 as shown in Table 1, for leading to optimal resolution of compounds, as well as to convenience regarding total time of analysis.

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, as shown in Table 2, taking into consideration the sufficient resolution, as well as the spectral criteria.

System suitability and Selectivity

The separation between compounds as shown in chromatogram presented in Fig. 2, is complete as signified from resolution factors R_t as well.

Table 1. Isocratic HPLC conditions investigated in the present study at a sensitivity setting at 0.002 aufs

Eluent	Percentage (v/v)	Flow Rate (ml/min)	Retention Time (min)		
			Bamifylline	AC-119	Caffeine
A:0.05M B	70:30	0.75	4.410	5.634	3.441
C:0.05M B	70:30	1.0	6.358	5.063	3.200
C:0.05M B	70:30	0.8	8.012	6.655	3.986
C:0.05M B	75:25	1.0	5.590	4.650	3.119
C:0.05M B	67:33	1.0	11.529	7.047	3.536
C:0.025M B	70:30	1.0	7.299	5.684	3.262
C:0.075M B	70:30	1.0	7.207	5.231	3.276
C:0.05M B	72:28	1.0	6.005	4.821	-
C:0.05M B	65:35	1.0	9.547	6.236	-
C:0.05M B	80:20	1.0	4.580	4.200	-
C:0.1M B	70:30	1.0	6.124	4.663	-
C:0.05M B	67:33	1.0	8.085	5.640	3.325
C:0.05M B	67:33	1.1	7.382	5.164	3.044
C:0.05M B	67:33	1.2	6.750	4.730	2.789
C:0.05M B	67:33	1.5	5.194	3.707	2.199
C:0.05M B	67:33	0.9	8.990	6,.323	3.651
C:0.05M B	67:33	0.8	10.120	7.037	4.092
C:0.05M B	67:33	0.75	10.466	7.450	4.358
C:0.05M B	67:33	0.70	11.107	7.931	4.661

A = CH_3CN

B = CH_3COONH_4

C = CH_3OH

Table 2. List of organic compounds tested as internal standards in the HPLC analysis of Bamifylline and AC-119

No	Compound	Notes
1	Xanthine	1
2	1,7-dimethylxanthine	2
3	1,3,9-trimethylxanthine	2
4	1-methylxanthine	2
5	3-methylxanthine	2
6	7-methylxanthine	2
7	8-methylxanthine	1
8	Theophylline	3
9	Theobromine	3
10	Tolfenamic acid	4
11	Flufenamic acid	5
12	Paracetamol	2
13	1,2-dihydroxyanthraquinone	6
14	Codeine	7
15	Caffeine	8

1. No absorbance at the analytical wavelength of bamifylline, 277 nm.
2. No resolution from solvent peak.
3. Better resolution than in case 2, but yet no sufficient.
4. Long retention time.
5. Same retention time as bamifylline, thus no separation.
6. Broad peak, inappropriate for use as internal standard.
7. No pure compound provided.
8. Sharp peak with sufficient resolution from solvent peak and analytes.

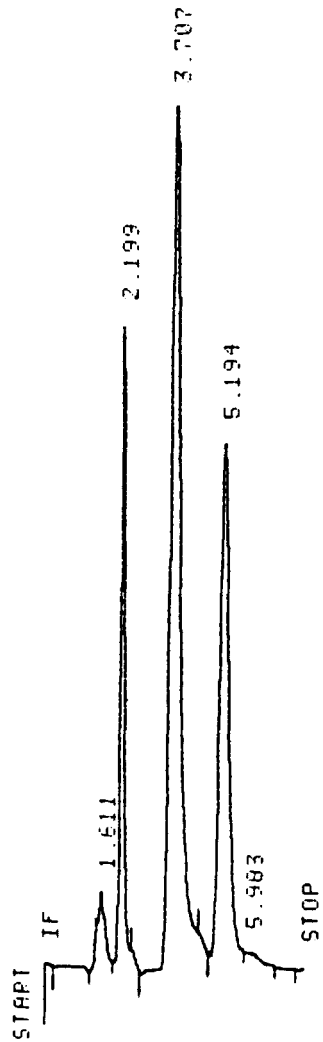


Fig 2. High-performance liquid chromatogram of Caffeine (2.199 min), AC-119 (3.707 min) and Bamifylline (5.194 min) using chromatographic conditions described in text. Concentration of Bamifylline and AC-119 is 3.0 ng/μl. Concentration of internal standard, Caffeine, is 0.784 ng/μl.

The resolution factor, R_t was calculated between the peaks from the equation:

$$R_t = 2\left[\frac{t_{R_2} - t_{R_1}}{W_1 + W_2}\right]$$

where t_{R_2} and t_{R_1} are the retention times of the two separated peaks, W_1 and W_2 are the peak widths at the base of the two respective peaks[10].

These were found to be 1.88 between caffeine and AC-119 and 1.49 between AC-119 and bamifylline, at a flow rate of 1.5 ml/min.

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.

Therefore the proposed method can be applied to the analysis of bamifylline and AC-119 in presence of caffeine, in pharmaceutical preparations (Fig. 3 and Fig. 4).

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 (LOQ) were calculated on the basis of regression equation data and found to be 1.15 and 2.90 ng for AC-119 and bamifylline respectively.

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 methanol.

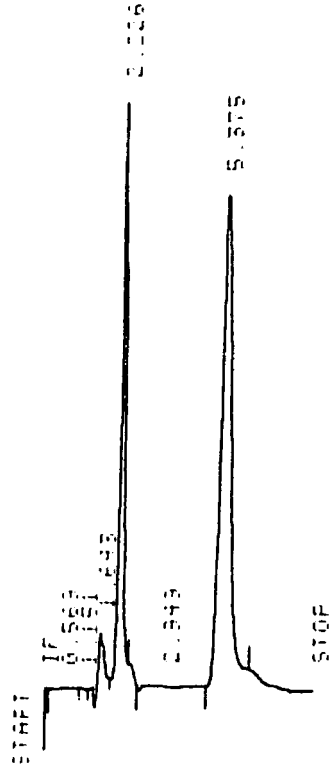


Fig. 3. HPLC chromatogram of Bamifylline 3.0 ng/ml in Pharmaceutical formulations: Tablets, with Caffeine, internal standard, 0.784 ng/µl. Chromatcgraphic conditions are described in text. Peaks: (2.226 min) Caffeine (5.375 min) Bamifylline.

Calibration curve was constructed in the presence of 0.784 ng/µl of internal standard at concentrations of 0.20 - 0.50 - 1.0 - 2.0 - 3.0 - 5.0 - 7.0 - 10.0 ng/µl for bamifylline and metabolite AC-119.

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.

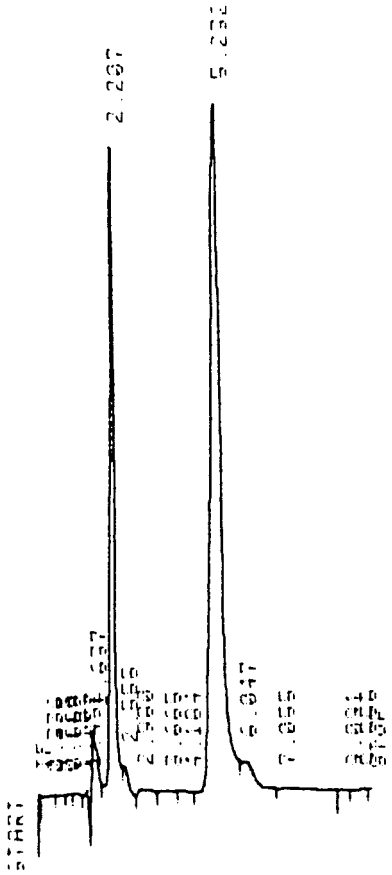


Fig. 4. HPLC chromatogram of Bamifylline 3.6 ng/μl in Suppositories with Caffeine, internal standard 0.784 ng/μl.
Chromatographic conditions are given in text.
Peaks: (2.207 min) Caffeine. (5.232 min) Bamifylline.

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

Sample pretreatment for analysis of Bamifylline in Pharmaceutical Preparations

Pharmaceutical formulations: tablets and suppositories, were treated as follows, in order to check the applicability of the developed HPLC method of bamifylline determination:

a. Tablets:

Ten tablets, containing 300 mg of bamifylline according to the label, were weighed and the average tablet weight was found to be 496.06 mg.

After finely powdering of the tablets in a porcelan mortar, a portion of 16.5 mg was quantitatively transferred into a 100 ml volumetric flask and diluted to volume with methanol after sonication. The concentration of this solution was namely 100 ng/μl. From this stock solution three working solutions were prepared by the proper dilution in order to obtain concentrations: 1.5-3.0 and 5.0 ng/μl. Working solutions contained the internal standard caffeine at a concentration of 0.784 ng/μl.

Aliquots of 20 μl were injected into HPLC analytical column.

b. Suppositories:

Six suppositories containing 750 mg of bamifylline as stated at the label were weighed and gave an average suppository weight of 2.9703 g. After incubation at 37°C for 5 min a quantity of 3.0008 g was dissolved in 50 ml of MeOH, sonicated for 5 min, and diluted to 250 ml with methanol in order to give a solution containing 3000 ng/μl of bamifylline. Three working solutions of 1.8-3.6 and 6.0 ng/μl in bamifylline containing the internal standard caffeine at a concentration of 0.784 ng/μl were prepared after the appropriate dilutions. Aliquots of 20 μl were analysed by HPLC.

RESULTS AND DISCUSSION

There are not many literature references concerning determination of bamifylline and AC-119, its major metabolite. Determination methods

Table 3. Statistical Evaluation of Analysis Data

Methanolic Bamifylline	$Y=(-7.7428 \cdot 10^{-2} \pm 0.052963) + (0.689988 \pm 0.011327)X$
Solutions	$r=0.9998$ LOQ=0.146 ng/μl
Methanolic AC-119	$Y=(0.613959 \pm 0.240009) + (0.688227 \pm 0.037115)X$
Solutions	$r=0.9992$ LOQ=0.058 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 = $\frac{t \cdot Sa}{b} + Sxi$ *where*

$$Sxi = \frac{So}{b} \cdot \sqrt{1 + \frac{1}{b} + \frac{(yi - \bar{y})^2)}{b^2(Sx^2 - n\bar{x}^2)}} \quad (11)$$

r = Correlation coefficient.

proposed are time consuming and require significantly large volumes of biological samples. In the existing literature nothing concerning the analysis of those components in pharmaceutical preparations is reported. The proposed method offers the possibility of short analysis time and the ability of being used in routine analysis of bamifylline in tablets and suppositories..

Total chromatographic analysis was approximately 5 min and low limits of detection and quantitation were achieved.

Table 3 shows the results of statistical evaluation of analysis data.

Table 4. Intracalibration analysis (within-day run) of Bamifylline and metabolite AC-119 (n=10) in methanolic solutions

Compound	k´	a´	Added (ng)	Found ± SD (ng)	RSD (%)
Bamifylline	2.404	1.682[1]	10.0	12.77 ± 0.79	6.18
			40.0	41.14 ± 1.10	2.66
			60.0	58.50 ± 0.64	1.10
			100.0	93.01 ± 4.79	5.14
AC-119	1.429	3.240[2]	11.7	11.77 ± 0.83	7.05
			46.8	43.26 ± 0.97	2.24
			70.2	77.45 ± 2.12	2.73
			117.0	109.95 ± 5.51	5.01
Caffeine	0.441				

1 Bamifylline/AC-119

2 AC-119/Caffeine

3 t_0 = 1.526 min, measured by injection of dilute solution of sodium nitrite.

Table 5. Inter-day reproducibility of Bamifylline and AC-119 analysis in the presence of Caffeine (n=12) in methanolic solutions

Compound	Added (ng)	Found ± SD	RSD (%)
Bamifylline	40.0	41.12 ± 1.46	3.54
	60.0	59.68 ± 1.40	2.34
	100.0	95.94 ± 3.33	3.47
AC-119	11.7	11.52 ± 1.07	9.28
	70.2	73.70 ± 2.78	3.78
	117.0	110.35 ± 5.24	4.75

Table 6. Experimental results for the analysis of Bamifylline in pharmaceuticals by RP-HPLC with Caffeine as internal standard

Sample	Labelled Amount (mg)	Found[a] (mg)	RSD (%)	Analysed Quantity (ng)	Found[b] (ng)	RSD (%)
Tablets	300	306.26 ± 24.38	7.96	30.0	33.16 ± 2.94	8.87
				60.0	63.22 ± 4.31	6.82
				100.0	96.56 ± 7.87	8.15
Suppositories	750	750.66 ± 38.86	5.18	36.0	40.40 ± 1.33	3.28
				72.0	72.29 ± 5.70	7.88
				120.0	116.32 ± 7.14	6.14

a Mean value of 24 determinations ± Standard Deviation

b Mean value of 8 determinations ± Standard Deviation

Experimental results in basis of repeatability during determination of bamifylline and AC-119 in the presence of caffeine as internal standard are shown in Table 4.

Inter-day reproducibility results of bamifylline and AC-119 determination in presence of caffeine are shown in Table 5.

The developed method was applied to the analysis of bamifylline in pharmaceuticals: tablets and suppositories.

The results obtained are given in Table 6.

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.

Pharmaceutical formulations of bamifylline can be analysed and quantitatively estimated with separation time of 5 min and good accuracy and precision.

Caffeine was proved to be a very suitable internal standard for this purpose.

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