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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF BAMIFYLLINE AND ITS THREE METABOLITES IN HUMAN PLASMA

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SUMMARY

The performance of a gradient elution normal-phase system and of two isocratic elution reversed-phase ion-pair systems was investigated for the high-performance liquid chromatographic separation of bamifylline and its three metabolites. Three packings and mobile phases were tested and the best separation was achieved using a reversed-phase ion-pair system with Hypersil ODS 3 μm as stationary phase. The effects of alkyl chain length and concentration of alkyl sulfonates, salt concentration, column temperature and competing amine on separation and peak tailing are discussed. The determination of bamifylline and metabolites in human plasma using the defined optimal chromatographic conditions is reported.

INTRODUCTION

Bamifylline* is a xanthine derivative obtained by bisubstitution of theophylline. The drug is used in the treatment of asthma and reversible airway obstructions. The pharmacokinetic profile and metabolism of the drug have been studied in man using various methods [1-3]. Three metabolites of bamifylline were identified (Fig. 1): AC 85, AC 155 and AC 119, but AC 85 and AC 155 were not separated. This paper describes three different high-performance liquid chromatographic (HPLC) systems we investigated in order to achieve optimal separation of bamifylline and its three metabolites.

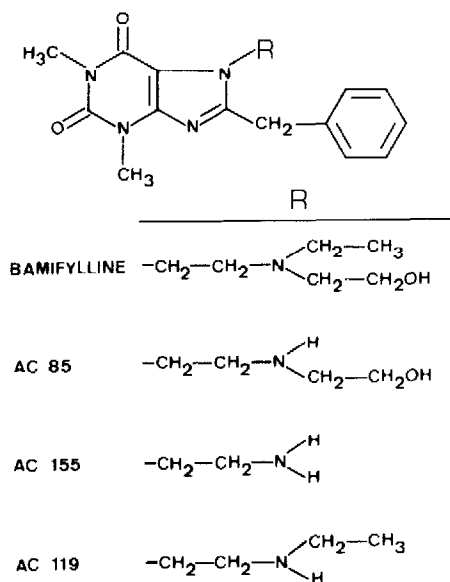


Fig. 1. Chemical structure of bamifylline and its metabolites.

Choice of the counter-ion size and concentration for the selected chromatographic procedure is discussed. The isocratic method developed here, using an internal standard, is found to be suitable for therapeutic determinations and pharmacokinetic investigations. Samples can be injected at 20-min intervals.

EXPERIMENTAL

Chemicals and reagents

Bamifylline and its metabolites AC 85, AC 155 and AC 119 were provided by Spret-Mauchant Laboratories (Spret-Mauchant, Gennevilliers, France). Standard aqueous solutions from 1.0 to 40 $\mu\text{g}/\text{ml}$ of each compound were extemporaneously obtained from stock solutions (100 $\mu\text{g}/\text{ml}$) prepared in methanol-water (50:50, v/v) and stored at +4°C in brown glass flasks for up to

*Trademark: Trentadil.

one month. The internal standard, fenetylline, was supplied by Gerda Laboratories (Gerda, Tassin, France); a 25- $\mu\text{g}/\text{ml}$ standard solution of fenetylline was prepared in methanol-water (50:50, v/v).

Acetonitrile for reversed-phase HPLC, potassium chloride and hydrochloric acid of "Titrisol" grade were respectively purchased from Prolabo (Prolabo, Paris, France) and from Merck (E. Merck, Darmstadt, F.R.G.). Pentane-, hexane-, heptane- and octanesulfonic acid sodium salts were provided by Fluka (Fluka, Buchs, Switzerland).

Chromatography

Different HPLC systems were studied using two different instrumentations:

(1) An isocratic system consisting of the following components: a Waters Model 6000 A pump (Waters, Paris, France); a Pye-Unicam spectrophotometer (Pye-Unicam, Paris, France) operated at 275 nm; a Rheodyne 7125 injection valve (Touzart et Matignon, Vitry, France) equipped with a 50- μl loop; the detector output was connected either to a Kontron W + W 610 recorder (W.W. Electronic Inc., Basel, Switzerland) or to a HP Model 3390 A integrator (Hewlett-Packard, Paris, France).

(2) A gradient system equipment consisting of a Model 1084 B Hewlett-Packard dual-head pump, equipped with a 79875 A variable-wavelength detector operated at 275 nm and a 79.850 B LC terminal.

Three different commercially available supports were used for the column packing: Nucleosil C18 (5 μm), Nucleosil NH_2 (5 μm) (Macherey-Nagel, Düren, F.R.G.) and Hypersil ODS (3 μm) (Shandon, Runcorn, U.K.). These chemically bonded silica gels were slurry packed in 15 cm \times 4.6 mm I.D. stainless-steel columns with a Haskel pump (Touzart et Matignon, Paris, France), according to a method described by Coq et al. [4] using *n*-butanol as the slurry medium and methanol as the packing solvent.

Three distinct mobile phases were tested with different stationary bonded phases and mobile phases. Their characteristics are given in Table I.

Sample preparation and plasma extraction

Venous blood samples (5 ml) were collected into 10-ml Vacutainer green-stoppered tubes (Becton-Dickinson, Missisauga, Canada) and centrifuged at 900 *g*. When the determination was not carried out immediately, the plasma was frozen at -20°C in plastic tubes. In these conditions, no degradation of drugs was noted after two months' storage.

A 2-ml volume of plasma was added to 200 μl of 2 *N* HCl (pH of the final mixture = 2.6), 100 μl of 25 $\mu\text{g}/\text{ml}$ internal standard solution and 7 ml of methylene chloride-ethyl ether mixture (4:7, v/v) in 10-ml Teflon-lined screw-capped glass tubes. Tubes were shaken for 10 min on a Laboral oscillating agitator (Prolabo, Paris, France) and then centrifuged at 900 *g* for 10 min. The upper (organic) layer was discarded, 100 μl of 5 *N* NaOH (final pH = 11.6) and 7 ml of methylene chloride-diethyl ether (4:7, v/v) were then added to the aqueous layer. Tubes were shaken for 10 min and spun at 900 *g* for 5 min. The upper organic phase was then collected in conical base glass tubes and evaporated to dryness under a very light nitrogen stream at

TABLE I

STATIONARY AND MOBILE PHASE CHARACTERISTICS FOR THE DIFFERENT CHROMATOGRAPHIC SYSTEMS

HPLC system	Stationary phase	Mobile phase	Time (min)	% B	Flow-rate (ml/min)
Normal-phase chromatography [1]	Nucleosil NH ₂ , 5 μm (Macherey-Nagel)	Pump A			1.5
		Isooctane—methylene chloride (20:80, v/v)			
		Pump B			
		Isopropanol	0.0	5.0	
			3.0	10.0	
			6.0	15.0	
			10.0	5.0	
Ion-pair reversed-phase chromatography [2]	Nucleosil C18, 5 μm (Macherey-Nagel)	Aqueous phase (pH=2.0)		73% (V)	1.2
		KCl 0.2 N (14.9 g/l) =25 ml			
		HCl 0.2 N=75 ml Distilled water=900ml Heptanesulfonate=1.5 g Acetonitrile		27% (V)	
Ion-pair reversed-phase chromatography* [3]	Hypersil ODS, 3 μm (Shandon)	Aqueous phase (pH=3.0)		78% (V)	1.3
		Distilled water=1000 ml Heptanesulfonate=1.5 g KCl=3 g 1 N Acetic acid to pH=3.0			
		Acetonitrile		22% (V)	

*This method must be considered as the definitive system.

35–40°C. The dry residue was redissolved in 200 μl of mobile phase and 50 μl were then injected into the chromatograph.

Calibration curves and calculation

Plasma samples were spiked with increasing amounts of bamifylline and each metabolite (final concentrations: 0.05, 0.1, 0.5, 1, 2 μg of each drug per ml of plasma) and 100 μl of 25 μg/ml internal standard solution. The samples were submitted to the extraction procedure described and standard curves were generated for each series of determinations by plotting peak height ratios (drug/internal standard) against known drug concentrations.

Plasma concentrations were interpolated from these standard curves. Accurate results in the range 0.01–2 μg/ml could also be alternatively obtained using a HP integrator; in this case, calibration was obtained from a 0.5 μg/ml standard solution.

RESULTS AND DISCUSSION

Chromatographic system development

Chromatograms obtained using the different HPLC systems previously

described are shown in Fig. 2. Poor selectivity was obtained using Nucleosil NH₂ (A₁) but separation was improved after impregnation of this packing with heptane sulfonate (A₂). Unfortunately, this system was unstable and resolution decreased quickly since no counter-ion could be pumped through the column using iso-octane—methylene chloride—*isopropanol* as mobile phase. In spite of the good selectivity observed, this system was rejected due to the insolubility of heptanesulfonate in the mobile phase. Using the ion-pair reversed-phase system with Nucleosil C18 (5 μm) as stationary phase, metabolites AC 85 and AC 155 were not separated, whatever alkylsulfonate counter-ion size or percentage of organic phase modifier we used. Hypersil ODS (3 μm) appeared to be a suitable alternative: a correct selectivity was obtained, although a concomitant and conflicting increase in peak broadening was noted. We were unable to explain this increase in peak broadening. In one paper, Cooke and Olsen [5] related conclusions of two authors indicating that sufficiently small particles may involve finite kinetics of solute transfer between stationary and mobile phases [6], or thermal effects due to high frictional flow resistance [7] and so negate any advantage using such packing materials. Karger et al. [8]

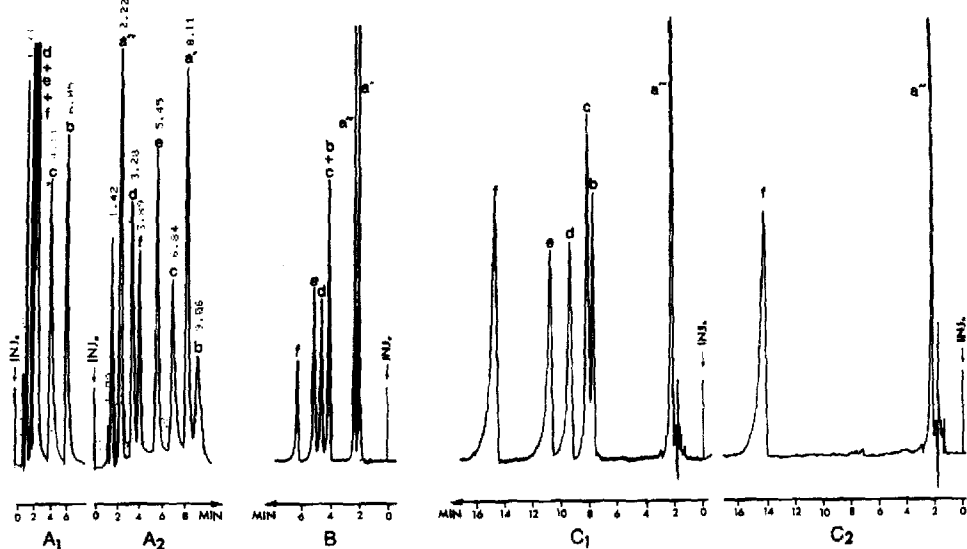


Fig. 2. (A) Chromatograms of a standard solution (in mobile phase) spiked with caffeine (a'), bamifylline (d), fenethylline (f), AC 119 (e), AC 155 (c) and AC 85 (b) at concentrations ranging from 5 to 10 μg/ml of each compound. These chromatograms were obtained before (A₁) and after (A₂) stationary phase was impregnated with counter-ion. Other chromatographic conditions are described in Table I [1]. UV wavelength = 275 nm. (B) Chromatogram of a standard solution (in mobile phase) spiked with theophylline (a'), caffeine (a''), AC 85 (b), AC 155 (c), bamifylline (d), AC 119 (e) and fenethylline (f) at 10 μg/ml for each compound. Chromatographic conditions are described in Table I [2]. UV wavelength = 275 nm. (C) Chromatogram (C₁) of a plasma extract spiked with AC 85 (b), AC 155 (c), bamifylline (d) and AC 119 (e), at 0.5 μg/ml for each compound and 1.25 μg/ml of fenethylline (f). This plasma contained caffeine (a'') at 3.2 μg/ml before spiking. Also chromatogram (C₂) of a blank plasma extract containing only fenethylline (f) at 1.25 μg/ml and caffeine (a'') at 3.2 μg/ml. In these two cases (spiked and blank plasma), chromatographic conditions are described in Table I [3]. UV wavelength = 275 nm.

reported that the most prevalent causes responsible for the poor peak shape observed when a secondary chemical equilibrium was used in the control of separation were competing side-reactions and slow kinetics in the chemical equilibrium steps. In this last case, band symmetry and efficiency can be improved by a change in temperature or in solvent conditions (ionic strength, etc.).

As will be seen later, the problem of peak tailing was partially resolved when potassium chloride (3 g/l) was added to the aqueous mobile phase.

The HPLC system 3 described in Table I allowed the separation of all the compounds of interest and was used for further studies.

Influence of the alkyl chain length and concentration of the pairing reagent on retention and selectivity of drug compounds

First of all the influence of counter-ion size on the retention of bamifylline and its metabolites was studied. Effects of pentane, hexane, heptane and octanesulfonate were successively investigated. As described in Fig. 3a, the capacity factor ($k' = (t_R - t_0)/t_0$) for a given mobile phase increased exponentially with the carbon number in the alkyl chain of the counter-ion. Investigation of selectivity factors (Fig. 3b) obtained from $\alpha = k'_y/k'_x$, where k'_y and k'_x are respectively the capacity factors of the y -th and x -th samples components (y being more retained than x), showed a relative improvement or constancy except in the case of α for bamifylline/AC 155, when increasing the alkyl chain length of the counter-ion. An hypothesis to explain this particular behaviour is that the accessibility of the ammonium group of bamifylline

$$\begin{array}{l} \text{---N---CH}_2\text{---CH}_3 \\ \quad \quad \quad \diagdown \\ \quad \quad \quad \text{CH}_2\text{---CH}_2\text{OH} \end{array}$$

is less than that of the other compounds. In agreement with the ion-pair mechanism proposed by Bidlingmeyer [9], i.e. the ion-pair model, it can be assumed that the formation of "ion-pair" is not complete between counter-ion and bamifylline. This difficulty to obtain an "ion-pair" may be especially important as the number of carbon atoms in the alkyl sulfonate chain length increases. Consequently, the retention of the ion-pair counter-ion-bamifylline increases less than that of the ion-pair counter-ion-AC 155.

Fig. 4. shows the effect of heptane sulfonate concentration in the mobile phase on capacity and selectivity factors. Concentrations from 0.1 to 2 g/l were successively used. According to the results achieved, a good compromise was obtained between retention time and selectivity factors of the different compounds by using a mobile phase containing 1.5 g/l heptanesulfonate. In these chromatographic conditions, AC 85 and AC 155 were separated with a resolution of less than 1, while the resolution between AC 155 and bamifylline, and between bamifylline and AC 119 was at least 1.0 and 1.2.

Influence of salt concentration, column temperature and competing amine on peak broadening and retention

When the KCl concentration in aqueous mobile phase was increased up to 3 g/l (Table II) to mask unreacted silanols, a decrease in capacity factors was

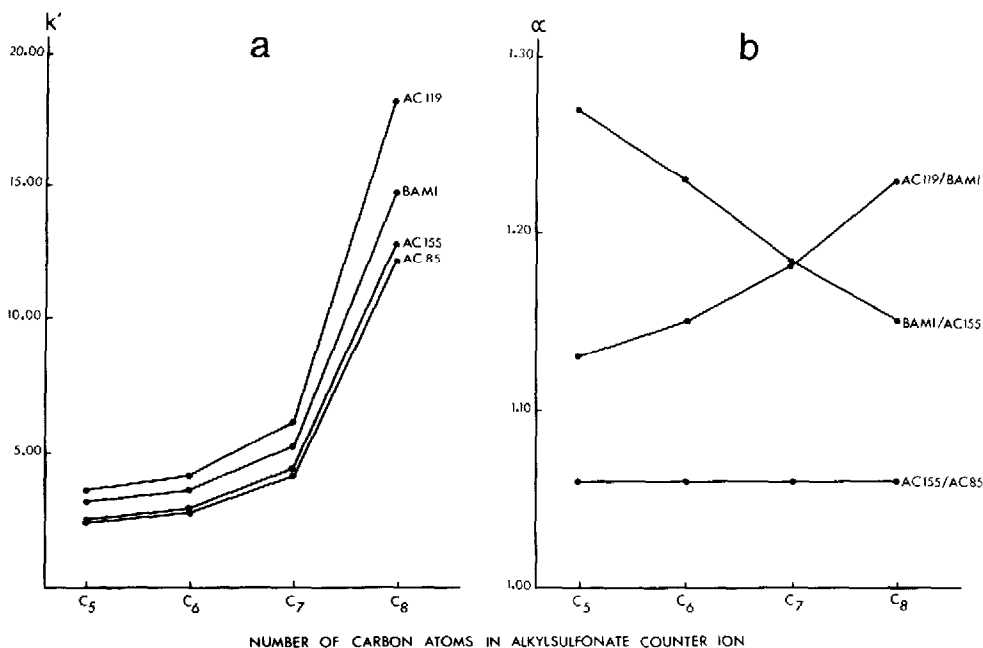


Fig. 3. Effect of counter-ion size on the k' values (a) and selectivity factor values α (b) for bamifylline and its metabolites. Column: Hypersil ODS, 3 μ m (150 \times 4.6 mm). Mobile phase: as described in Table I [3].

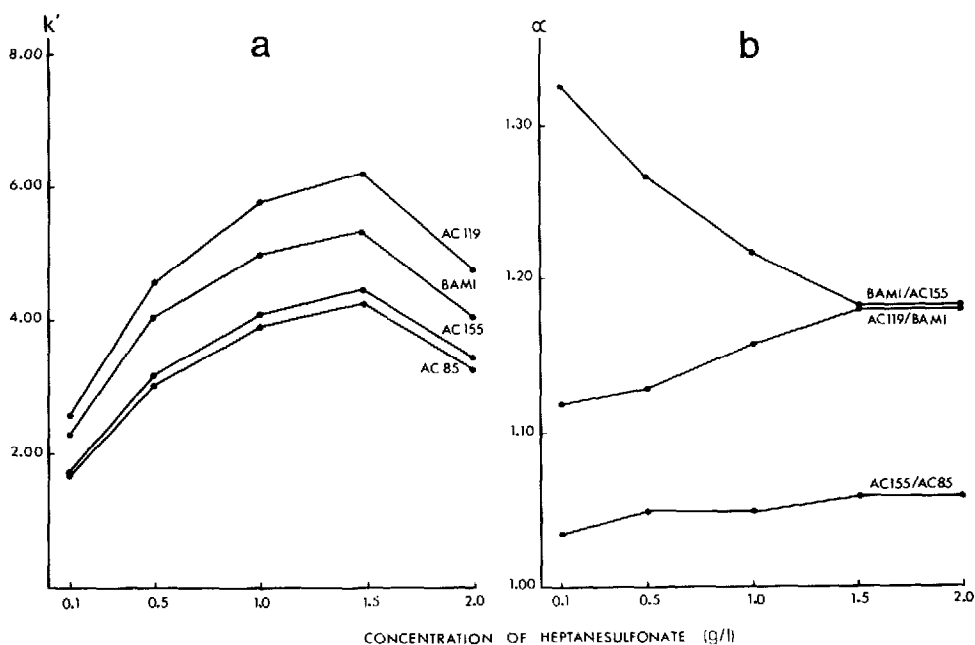


Fig. 4. Effect of heptanesulfonate concentration on the k' values (a) and selectivity factor values α (b) for bamifylline and its metabolites. Column: Hypersil ODS, 3 μ m (150 \times 4.6 mm). Mobile phase: as described in Table I [3].

TABLE II

EFFECT OF KCl OR Na₂SO₄ CONCENTRATION IN AQUEOUS MOBILE PHASE ON NUMBER OF THEORETICAL PLATES (*N*), CAPACITY FACTOR (*k'*), SELECTIVITY FACTOR (α) AND PEAK ASYMMETRY*

Chromatographic conditions (except for KCl or Na₂SO₄ concentrations) are described in Table I [3].

Salt conc. (g/l)	AC 85		AC 155		Bamifylline		AC 119		α					
	<i>N</i>	<i>k'</i>	<i>N</i>	<i>k'</i>	<i>N</i>	<i>k'</i>	Asym.	<i>N</i>	<i>k'</i>	Asym.	AC 155 AC 85	Bamifylline AC 155	AC 119 Bamifylline	
KCl	0	2830	7.10	2800	7.70	790	12.0	2.7	540	14.0	0.9	1.08	1.56	1.17
	1	11025	2.73	8940	2.89	8695	3.71	4.5	3500	4.33	5.3	1.06	1.28	1.17
	2	12720	2.13	11025	2.27	6100	2.82	2.5	6080	3.33	2.75	1.07	1.24	1.18
	3	14400	1.93	12280	2.04	7260	2.51	2.0	7250	2.98	2.5	1.06	1.23	1.19
	4	8980	3.10	8200	3.28	6980	3.94	2.5	6900	4.63	4.3	1.05	1.20	1.17
Na ₂ SO ₄	3	6720	2.64	4520	2.82	1160	4.0	Tailing peak	1020	4.73	Tailing peak	1.07	1.42	1.18

*Measured at 10% of peak height.

observed, and peak asymmetry (measured at 10% of the peak height) was reduced but remained still higher than that obtained with Nucleosil C18 (5 μm), 2.0 versus 1.0, for bamifylline. Selectivity factors were unchanged or decreased (bamifylline/AC 155) and theoretical plates were enhanced. When KCl concentrations higher than 3 g/l were used these global improvements disappeared. Substitution of 3 g/l KCl by 3 g/l Na_2SO_4 in aqueous mobile phase did not give satisfactory results. It is common knowledge that the chloride ion may be detrimental to the HPLC instrument; however, as the use of chloride ion resulted in good chromatographic separations, and as no damage to the chromatographic apparatus occurred after a six-month work period, we suggest that the chloride ion may be used in the mobile phase. Nevertheless, we advise users to wash the HPLC instrument carefully, pumping through at least 50 ml of distilled water once a week.

The effect of temperature was studied after the column was set in a chromatograph oven at temperatures from 30 to 65°C. We noted that selectivity factors were enhanced between AC 155 and bamifylline (1.23 up to 1.41) but decreased between bamifylline and AC 119 (1.19 down to 1.13) and between AC 85 and AC 155 (1.06 down to 1.00).

Improvement in peak shape was observed when *n*-nonylamine was added to aqueous mobile phase (with or without 3 g/l KCl) at concentrations ranging from $1.3 \cdot 10^{-4}$ to $2.0 \cdot 10^{-3}$ M, but a simultaneous decrease of resolution appeared.

Both of these modifications (temperature or competing amine) were then discarded.

Linearity, sensitivity, specificity

Standard curves were obtained by measuring the peak height ratios (drug/internal standard) on chromatograms obtained from drug-free plasma spiked with bamifylline, its metabolites and fenetylline. Linear curves were observed when plotting peak height ratios versus concentration (0.01, 0.1, 0.2, 0.5, 1, 2 μg of each compound per ml). Each value was the mean of six measurements. The calibration curves for AC 85, AC 155, bamifylline and AC 119 could be respectively expressed by the following equations: $Y = 0.981X$ ($r = 1.00$), $Y = 1.01X$ ($r = 1.00$), $Y = 0.986X$ ($r = 1.00$) and $Y = 0.992X$ ($r = 1.00$). The detection limit (signal/background = 3) was 0.01 $\mu\text{g}/\text{ml}$ for each compound. No interference with endogenous components (uric acid, creatinine) or with tested drugs (10 $\mu\text{g}/\text{ml}$ each in plasma) such as clobutinol, almitrine, bromhexine, troleandomycin, doxycycline, furosemide, salbutamol, altizide, spironolactone, canrenoate potassium and eprazinone was found.

Reproducibility, recovery

Within-day reproducibility was determined by carrying out fourteen determinations of plasma spiked with 0.2, 0.5 and 1.0 $\mu\text{g}/\text{ml}$ of bamifylline and each metabolite. Day-to-day reproducibility was obtained by carrying out every day and during ten days a determination from plasmas spiked with 0.5 $\mu\text{g}/\text{ml}$ of each drug compound. Coefficients of variation (Table III) were respectively less than 8.2% and less than 6.3%.

TABLE III

WITHIN-DAY AND DAY-TO-DAY REPRODUCIBILITY OF THE METHOD

	Drug concentration ($\mu\text{g/ml}$)	Coefficient of variation (%)			
		AC 85	AC 155	Bamifylline	AC 119
Within-day ($n = 14$)	0.2	6.92	8.17	4.71	6.71
	0.5	5.27	5.14	4.17	5.61
	1.0	4.88	6.30	4.54	6.94
Day-to-day ($n = 10$)	0.5	6.22	4.97	4.13	6.30

A recovery study was carried out by adding a known amount of bamifylline and metabolites to a drug-free plasma sample at two concentrations (0.5 and 1.0 $\mu\text{g/ml}$). After extraction, the dry residue was dissolved in a mobile phase containing an internal standard amount equivalent to a 100% extraction yield. For each concentration, five extractions were performed; means of peak height ratios were computed and compared to the peak height ratio of an injected amount of drug compound and internal standard equivalent to a 100% extraction yield. As can be noted from Table IV, recovery percentages are greater for bamifylline than for its metabolites.

TABLE IV

RECOVERY DATA FOR ASSAY OF BAMIFYLLINE AND ITS METABOLITES

Plasma concentration ($\mu\text{g/ml}$)	n	Mean recovery (% \pm S.D.)			
		AC 85	AC 155	Bamifylline	AC 119
0.5	5	62.6 \pm 3.0	75.2 \pm 3.4	94.2 \pm 3.5	72.4 \pm 3.6
1.0	5	61.6 \pm 2.3	71.4 \pm 3.9	89.6 \pm 2.7	70.6 \pm 2.1

Clinical applications

A typical chromatogram obtained, using the method previously described, from a subject treated for three months with bamifylline is illustrated in Fig. 5. The chromatographic separation of bamifylline and its metabolites was achieved in less than 15 min. The retention times (t_R) of AC 85, AC 155, bamifylline, AC 119 and internal standard were 6.8, 7.2, 8.3, 9.4 and 12.6 min, respectively. This chromatogram shows two unidentified peaks (unknown minor metabolites?) at $t_R = 8.0$ min (X) and $t_R = 11.2$ min (Y).

Preliminary results of plasma concentration of bamifylline and metabolites were obtained from ten subjects receiving orally 600 mg of Trentadil per day (2×300 mg) for at least one month. Mean plasma concentrations at the trough (just before the next-morning dose) and at nearly the peak level (60 min after

oral administration) were, respectively, 0.16 and 0.43 $\mu\text{g/ml}$ for AC 85, 0.12 and 0.12 $\mu\text{g/ml}$ for AC 155, 0.34 and 1.28 $\mu\text{g/ml}$ for bamifylline, 0.21 and 0.28 $\mu\text{g/ml}$ for AC 119.

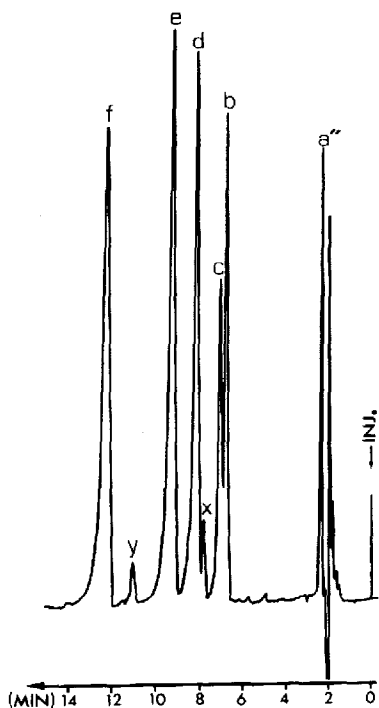


Fig. 5. Chromatogram of a plasma extract from a patient receiving a 600-mg oral dose of bamifylline (Trentadil) for three months and spiked with 1.25 $\mu\text{g/ml}$ of fenetylline (internal standard). Chromatographic conditions are described in Table I [3]. a'' = caffeine, b = AC 85, c = AC 155, d = bamifylline, e = AC 119, f = fenetylline, x and y = unknown peaks (minor metabolites?).

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