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Micro-scale liquid chromatographic method for the determination of bamifylline and its major metabolite in human plasma

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ABSTRACT

A sensitive micro-scale method based on isocratic elution reversed-phase ion-pair chromatography for the determination of bamifylline and its major metabolite (AC 119) in human plasma is described. The method is based on a liquid–liquid extraction clean-up followed by analysis on an LC-Packings Fusica (Delta Pak, RP-18, 5 μm, 300 Å) column (15 cm × 330 μm, I.D.) with 0.03 *M* heptanesulphonate (pH 3.5)–acetonitrile (7:3, v/v) as the mobile phase. Data with respect to recovery, reproducibility and limits of detection are reported and discussed.

INTRODUCTION

Bamifylline (Fig. 1a), a 7,8-disubstituted derivative of theophylline with bronchodilatator properties, is used in the treatment of asthma and reversible airway

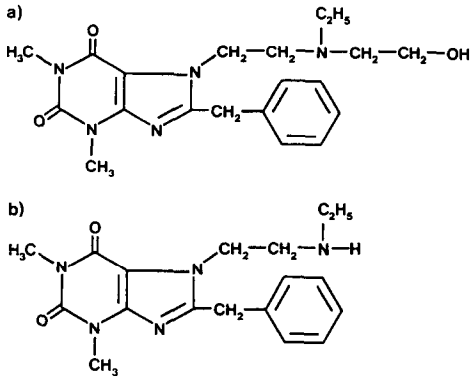

Fig. 1. Structures of (a) bamifylline and (b) metabolite AC-119.

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obstructions. The drug exhibits distinct metabolic and pharmacokinetic charcteristics, which differ markedly from those of theophylline. In earlier investigations on the pharmacokinetic behaviour of bamifylline, only a sophisticated mass fragmentographic technique was able to determine the very low plasma concentrations in the terminal range of the elimination curve.

The aim of this study was to develop a sensitive micro-scale method based on isocratic elution reversed-phase ion-pair chromatography to detect, in the nanomole range, bamifylline and its major metabolite (AC 119) (Fig. 1b) in human plasma.

EXPERIMENTAL

Samples

Four young, healthy volunteers (two men and two women) with a mean age of 27.3 ± 2.5 years, mean weight 65.4 ± 10.4 kg and mean height 170.4 ± 6.4 cm took part in the study. All the subjects received 300 mg of bamyfylline twice a day for 6 days (a total of 12 doses of 600 mg). Blood specimens for drug monitoring were obtained 1, 2, 4, 8 and 10 h after the first and at the last dose and the end of each dosing period (12th hour).

Sample preparation

A simple liquid–liquid extraction technique was used for both clean-up and extraction [1]. To a 1-ml sample of plasma in a 10-ml Teflon-lined screw-capped glass tube were added 0.2 ml of 2 *M* HCl and 4 ml of dichloromethane–diethyl ether (4.7, v/v). After vortex mixing and centrifugation (10 min, 1000 *g*), the upper layer was discarded and 0.1 ml of 5 *M* sodium hydroxide solution and 5 ml of dichloromethane–diethyl ether (4:7, v/v) were added to the aqueous layer. The tubes were shaken and then centrifuged at 1000 *g* for 10 min. The upper phase was carefully transferred into a 10-ml conical vial and the organic solvent was evaporated to a volume of *ca.* 0.5 ml under a stream of nitrogen at 35°C. The residue was then transferred into a 0.5-ml conical vial and evaporated to dryness. The dry residue was reconstituted in 100 μl of methanol.

Extraction efficiency

Spiked plasma samples ($n=4$) containing 60, 90, 120 and 150 ng/ml of bamifylline were extracted according to the procedure described under *Sample preparation*. The peak areas in these samples were then compared with those obtained from unextracted bamifylline standard solutions with comparable concentrations.

Standard solutions

Bamifylline and AC 119 (metabolite of bamifylline) standards were provided by Alfa Farmaceutici (Bologna, Italy) and were used without further purification. Stock solutions of bamifylline and AC 119 (150 μg/ml) were prepared by dissolving 215 mg of each standard, exactly weighed on a Cahn Model G2 electrobalance, in water purified using a Water I system (Gelman Ann Arbor, MI, U.S.A.) and diluted to volume in a 100-ml volumetric flask.

Apparatus

The liquid chromatographic (LC) system consisted of the following components: a Carlo Erba (Milan, Italy) Phoenix 20 CU micro-pump, a Kontron (Zurich, Switzerland) Model 433 UV capillary detector, equipped with an ultrasensitive UV flow cell (total volume 90 nl, optical path length 20 mm), a Valco (Houston, TX, U.S.A.) Model C14W injector with a 200-nl internal loop and an HP 9000 Series 300 work-station (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with an HP 7440A plotter.

Chromatographic conditions

A Fusica (Delta Pak, RP-18,5 μm, 300 Å) column (15 cm × 330 μm I.D.) (LC-Packings international, Amsterdam, The Netherlands) was used. The column was connected directly to the injector, and the column outlet capillary was connected directly to the flow cell via a small piece of Teflon tubing (Teflon tubing kit, TF-K1, LC-Packings International).

The separations reported were achieved under the following conditions: mobile phase, 0.03 *M* heptanesulphonate (pH 3.5)–acetonitrile (70:30, v/v); flow-rate, 8 μl/min; chart speed, 0.5 cm/min; temperature, 20°C; and detection wavelength, 210 nm.

The amounts of bamifylline and AC119 were calculated by applying the external standard method.

RESULTS AND DISCUSSION

Mobile phase selection was crucial for achieving the desired sensitivity and resolution. In preliminary experiments various C_5–C_8 alkanesulphonates were investigated as ion-pair reagents. The relationship between the chain length of the alkanesulphonate and the retention of bamifylline and AC 119 was studied. For a given mobile phase, as the chain length of the pairing reagent increased, so did the capacity factor (k'). In agreement with reported data [1], the influence of counter-ion

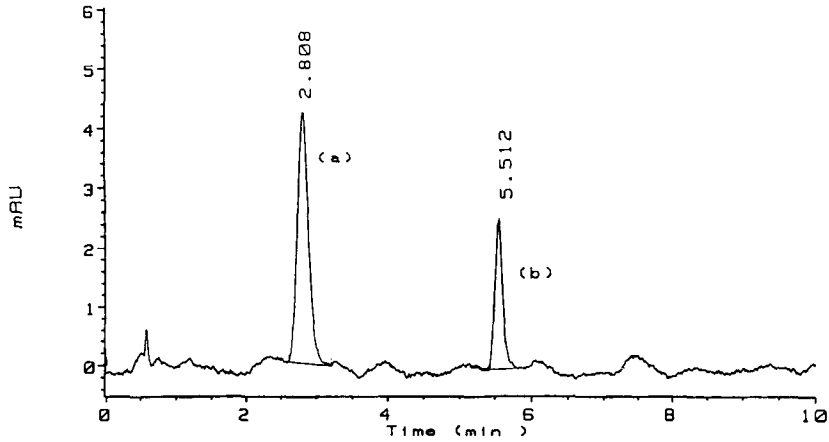

Fig. 2. Chromatograms of (a) bamifylline (20 ng) and (b) AC 119 (15 ng) standards. For conditions, see text.

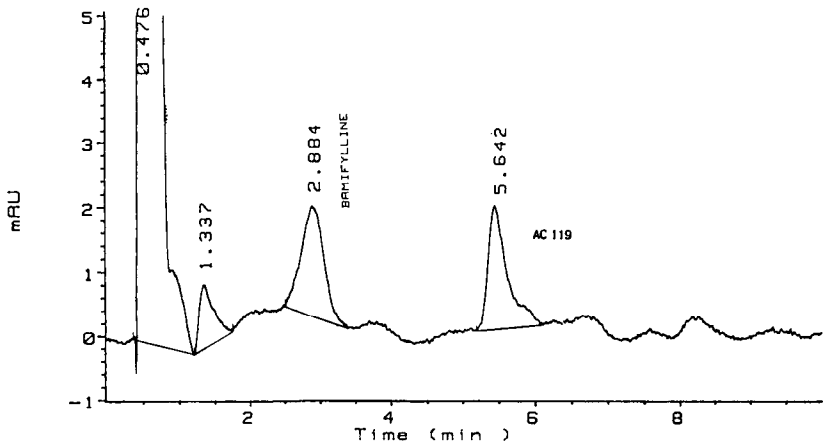

Fig. 3. Chromatogram of bamifylline and AC119 in plasma, 12 h after the oral administration of a 600-mg dose of bamifylline. For conditions, see text.

size on the selectivity factor (α) for bamifylline and AC119 showed a relative improvement with an increase in the chain length of the pairing reagent. Based on the selectivity and the total analysis time, heptanesulphonate was selected as the ion-pair reagent in the mobile phase. Preliminary experiments did not reveal that the presence of salts (potassium clhoride or sodium sulphate) in the mobile phase resulted in better resolution and peak asymmetry.

Example of chromatograms resulting from the LC assay of bamifylline and AC119 in plasma are shown in Figs. 2 and 3. With the solvent system used, the Fusica column was found to give sharp, symmetrical peaks and good selectivity. No interference with the retention times of the analyte peaks due to endogenous components was observed. In agreement with published data [2,3], the highest plasma levels of bamifylline were always reached about 1 h after dosing; 18 h after the last dose, the plasma concentrations of bamifylline and AC119 showed variations in each subject; the lowest value detected was 12 ng/ml.

The overall precision of the retention time was studied with respect to run-to-run and day-to-day variations. The run-to-run precision [relative standard deviation (R.S.D.)] for 20 runs within a single day averaged 1.66% and the day-to-day precision over a 3-week period with the same column was slightly higher, *ca.* 2.75%.

Calibration graphs were obtained by analysing spiked plasma samples over bamifylline and AC 119 concentration ranges of 30–900 and 40–1000 ng/ml, respectively. The least-squares regression fit showed good linearity (correlation coefficient = 0.998) for both bamifylline and AC 119. The intra- and inter-assay recoveries of bamifylline from spiked plasma sample were 90.3% with an R.S.D. of 2.8% and 92.1% with an R.S.D. of 3.4% (both 250 ng/ml, $n = 10$), respectively. The detection limits, with a signal-to-noise ratio of 3, were 12 ng for bamifylline and 9 ng for AC 119.

A storage stability study showed a slow decrease in bamifylline concentrations in both refrigerated and frozen plasma samples. The refrigerated sample showed little

loss after 4 weeks, but by week 8 the concentration of bamifylline in these samples had decreased by 11–15%. The same trend was observed with frozen plasma, but a significant decrease in bamifylline was found later (week 10).

In conclusion, the proposed method offers high selectivity and sensitivity for the determination of bamifylline and its major metabolite AC 119 in human plasma. This LC method, with a simple clean-up step, is suitable for pharmacokinetic studies.

ACKNOWLEDGEMENT

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