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Note

High-performance liquid chromatographic determination of 1-(3-butoxy-2-carbamoyloxypropyl)-5-ethyl-5-phenyl-(1*H*,3*H*,5*H*)-pyrimidine-2,4,6-trione in human plasma

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1-(3-Butoxy-2-carbamoyloxypropyl)-5-ethyl-5-phenyl-(1*H*,3*H*,5*H*)-pyrimidine-2,4,6-trione (BCPT) (Fig. 1) is a new thymoanaleptic drug currently used in the treatment of dysphoric states and mood disturbances. It is particularly suitable for use in geriatrics, especially in mental and related disorders in elderly people [1]. Recent investigations showed that a serotoninergic and a gabaergic system were involved in the mechanism of action [2]. The drug is rapidly absorbed from the gastrointestinal tract and undergoes extensive metabolism in the liver, with the formation of numerous metabolites [3,4]. Owing to the first-pass effect the concentration of BCPT in plasma is very low. Therefore a sensitive and selective high-performance liquid chromatographic (HPLC) method was developed, suitable for monitoring and pharmacokinetic trials.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile was of Spectrar quality from Mallinckrodt (Paris, KT, U.S.A.). Potassium carbonate of Optipur quality and chloroform of Uvasol quality were supplied by Merck (Darmstadt, F.R.G.). Sodium carbonate, sodium hydroxide, hydrochloric acid and disodium tetraborate 10-hydrate were of analytical grade and purchased from Fluka (Buchs, Switzerland). BCPT and internal standard were synthesized and purified as described earlier [5].

Borate buffer was prepared by mixing 89.2 ml of 0.05 *M* sodium carbonate and 10.8 ml of 0.05 *M* disodium tetraborate. Water was double-distilled in glass.

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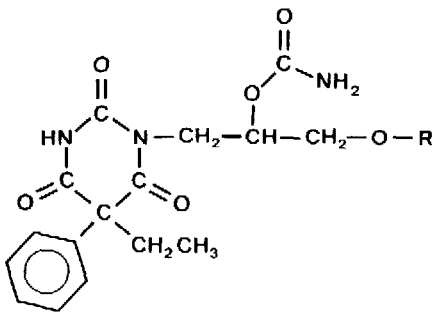

R

-butyl : BCPT

-amyl : internal standard

Fig. 1. Structures of BCPT and internal standard.

Instruments

The HPLC system consisted of a Model 6000 A solvent-delivery system from Waters Assoc. (Milford, MA, U.S.A.), a Waters U6K injector, a Spectroflow 773 UV detector from Kratos Analytical Instruments (Ramsay, NJ, U.S.A.) and a Model LCI-100 laboratory computing integrator from Perkin-Elmer (Norwalk, CT, U.S.A.). A short (2 cm) polymer PRP-1 10-μm column (Hamilton, Bonaduz, Switzerland) was connected between the pump and the injector to remove solvent impurities.

Solutions and calibration standards

Stock solutions of BCPT and of internal standard were prepared in triplicate, each containing ca. 7 mg per 500 ml in acetonitrile. Working standard solutions were prepared by appropriate dilution with acetonitrile. All vessels used for this operation were calibrated by weighing.

Extraction procedure

To 250 μl of plasma in a 2-ml Reacti-Vial (Pierce Eurochimie, Oud-Beijerland, The Netherlands) was added 0.5 ml of tetraborate buffer solution (pH 10.55), then 7.0 μl of internal standard solution and 1.0 ml of chloroform. The mixture was shaken for 10 min with a Vortex-Genie mixer and then centrifuged for 20 min at 3500 *g*. If an emulsion that formed during shaking persisted after centrifugation, vials were frozen at −20°C for 60 min and recentrifuged. The chloroform layer was transferred to a second 2-ml Reacti-Vial, and 0.2 ml of 0.1 *M* sodium hydroxide was added. The mixture was shaken for 10 min with the Vortex and centrifuged for 20 min at 3500 *g*. The chloroform phase was removed and discarded, and 40 μl of 1 *M* hydrochloric acid were added followed by 1.0 ml of chloroform. The phases were vortexed for 10 min and centrifuged for 5 min at 2200 *g*, and the chloroform layer was transferred to a 1-ml Reacti-Vial and evap-

orated under a stream of nitrogen to dryness. Before analysis, extracts were dissolved in 10 μl of mobile phase.

For calibration purposes 250 μl of human plasma (Zentrallaboratorium, Blutspendedienst SRK, Bern, Switzerland) were spiked with appropriate volumes of standard solutions and extracted as described above.

Chromatographic conditions

A 150 mm $\times$ 4.1 mm I.D. polymer PRP-1 5-μm column (Hamilton) was operated at 70°C at a flow-rate of 1.4 ml/min, corresponding to a pressure of ca. 135 bar. The mobile phase consisted of 19% acetonitrile and 0.05 *M* potassium carbonate (pH 11.45). Components were filtered separately through Millipore membrane filters (0.45 μm) before mixing. The mobile phase was degassed by helium before use. The peaks were detected at 254 nm, and the sensitivity was 0.0075 and 0.002 a.u.f.s. in the concentration range 10–30 ng/ml, respectively.

Analysis of results

The peak-area ratio (drug versus internal standard) was calculated for all samples. The calibration curves were calculated with the use of unweighted linear regression of peak-area ratios and the respective concentrations of calibration samples. Concentrations of BCPT in test samples were calculated using calibration graphs.

Two concentration ranges were investigated: 10–100 ng/ml with four calibration points and 100–13 000 ng/ml with seven calibration points. Each point corresponds to the mean concentration of BCPT at least in ten samples.

RESULTS AND DISCUSSION

The ionization of a molecule often represents the easiest way to enhance absorptivity, and consequently improve the limit of detection. This reaction is generally rapid and complete within few seconds and does not require an extra sample preparation step. This procedure was used for the determination of barbiturates separated in their acid forms on an octadecylsilane reversed phase, and ionized after passing through the column [6–8]. We investigated this procedure for the determination of BCPT in human plasma, but the instability of the baseline did not allow us to develop a reliable method. The separation of anionic forms of barbiturates was achieved with the use of a strong anion-exchange resin [9] and later with the use of styrene–divinylbenzene copolymer, PRP-1, which is compatible with alkaline eluents [10]. Using this phase under the chromatographic conditions described, we separated the anionic forms of BCPT and internal standard. A lower efficiency of this phase was compensated more than sufficiently by higher selectivity.

The first approach to the determination of BCPT in plasma was based on a precolumn enrichment with direct injection of filtered plasma into the HPLC system, using a 150 mm $\times$ 2 mm I.D. PRP-1 10-μm column packed in the laboratory. This method was simple, rapid and sensitive. However it had to be aban-

doned because of changes in selectivity due to the non-uniform quality of the early PRP-1 bulk resin.

Typical chromatograms obtained from human plasma extracts are shown in Fig. 2. The retention times of BCPT and internal standard were 6 and 12 min, respectively. For practical reasons only one diastereoisomer of the internal standard was used.

Selectivity and stability

The difference in lipophilicity between the parent drug and its hydroxylated metabolites was sufficient for baseline separation on the 150-mm PRP-1 column. Under the chromatographic conditions described, each of 22 metabolites was eluted earlier than BCPT. No interferences from normal plasma constituents were observed. The possibility of degradation of BCPT and internal standard during the chromatographic procedure was checked by repeated injection ($n=10$) of standard solutions at three different column temperatures (65, 70 and 75°C). Neither degradation products nor changes in peak areas were found.

Linearity and sensitivity

Calibration curves were obtained by plotting the peak-area ratios against the respective concentrations of BCPT in spiked plasma. An excellent linear rela-

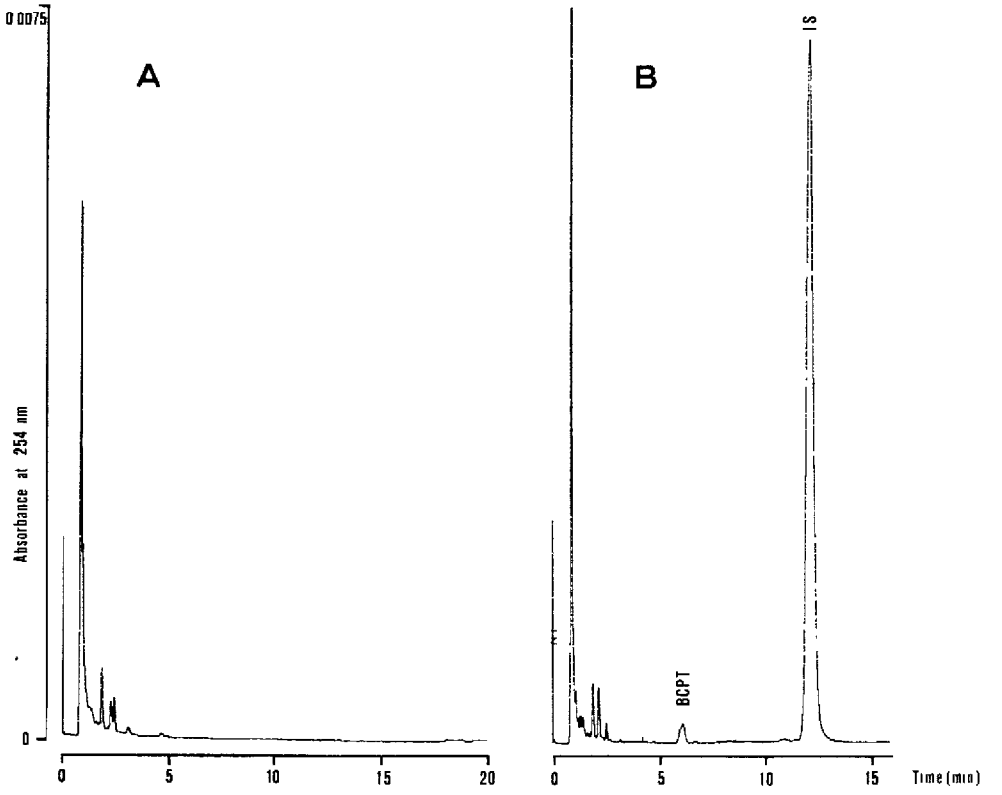

Fig. 2

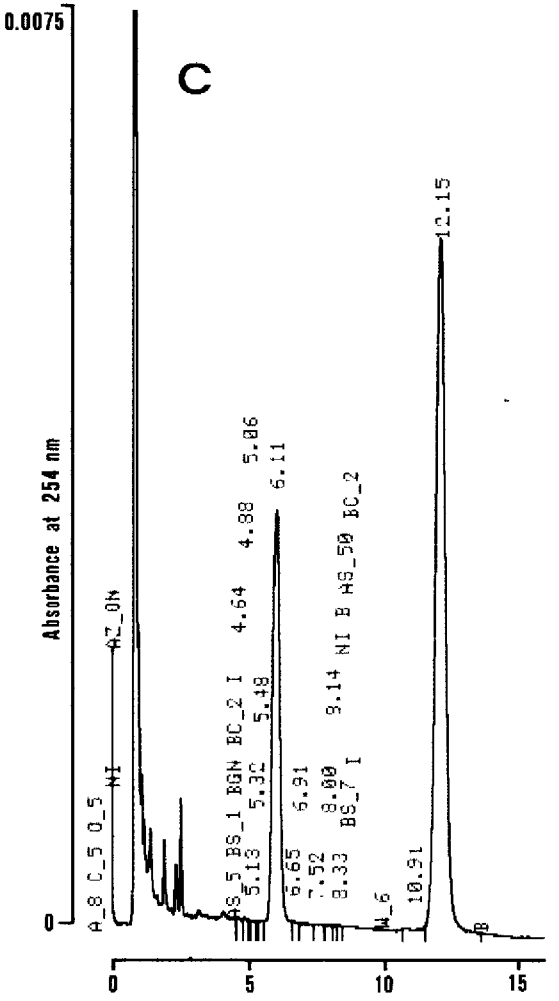

Fig. 2. Typical chromatograms obtained after the analysis of BCPT in human plasma extracts. (A) Blank plasma sample; (B) plasma sample spiked with 50 ng/ml BCPT and 2120 ng/ml internal standard (IS); (C) plasma sample obtained from a subject 4 h after a 600-mg oral dose of BCPT.

tionship was observed over both concentration ranges. Parameters of regression lines $y = bx + a$, where x is the peak-area ratio and y is the concentration in ng/ml, are shown in Table I. Each point represents a mean of at least ten samples. The limit of detection of BCPT was 5 ng/ml (signal-to-noise ratio greater than 2).

Precision and accuracy

The precision of the method was calculated from repeated analysis of control plasma containing BCPT at concentrations corresponding to the top, middle and bottom of each calibration range used. The exact concentration in samples was not known to the analyst. The results (Table II) demonstrate good reproducibility and accuracy in both concentration ranges.

TABLE I

CORRELATION BETWEEN PEAK-AREA RATIOS AND RESPECTIVE CONCENTRATIONS OF BCPT IN CONTROL PLASMA EXTRACT

Concentration of internal standard was 2120 ng/ml in both concentration ranges.

Concentration (y) (ng/ml)	n	BCPT peak-area ratio (x)		Calibration graph
		Mean $\times 10^3$	S.D. $\times 10^3$	
10.41	15	5.577	0.265	$y=2222.462x-1.943$
20.83	13	10.253	0.360	$r^2=0.999998$
41.66	12	19.582	1.059	
98.48	13	45.198	2.282	
98.48	12	44.219	1.741	$y=2200\ 763x-0.537$
307 74	12	140.120	1.627	$r^2=0.999970$
615.48	12	275.649	4.941	
1230.96	10	552.609	8.137	
2523.47	12	1145.736	13.451	
6613.32	15	3030.160	45.593	
12 91.72	16	5855.371	105.374	

TABLE II

PRECISION AND ACCURACY OF BCPT MEASUREMENTS IN SPIKED PLASMA

Amount added (ng/ml)	n	Amount found (ng/ml)	Difference (%)	S.D.	Coefficient of variation (%)
12 39	13	12.46	+0.56	1.95	15.68
20.83	12	21.07	+1.15	1.65	7.85
49.24	11	48.34	−1.83	2.20	4.46
83.32	12	83.63	+0.37	1.71	2.05
221.12	12	221.77	+0.29	2.67	1.21
492.38	12	491.04	−0.27	4.32	0.88
1007.74	12	1015.42	+0.76	15 29	1.51
5038.72	12	5068.83	+0.60	63.12	1.25
10 392.36	12	10 392.40	−0.61	131.67	1.27

Application of the method

This method was applied to a pharmacokinetic study of healthy volunteers. Fig. 3 shows the time course of BCPT plasma concentration after a single oral dose of a 600-mg BCPT tablet. The method was also applied to the therapeutic monitoring of BCPT plasma levels in clinical studies.

CONCLUSION

An HPLC method suitable for the quantitation of BCPT in human plasma during pharmacokinetic studies over several drug half-lives has been developed.

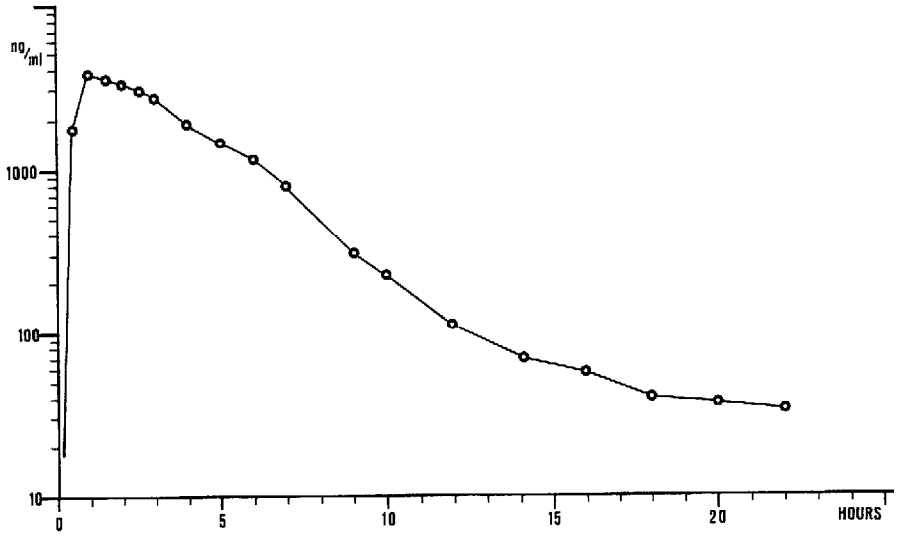

Fig. 3. Plasma concentration–time profile of a healthy volunteer following a single oral dose of 600 mg of BCPT.

The method is sufficiently selective and sensitive to allow precise and accurate determination of BCPT under therapeutic conditions.

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