Journal of Chromatography, 272 (1983) 406—410
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1520

Note

High-performance liquid chromatographic determination of ethyl biscoumacetate in human plasma

MITRA ARMAN and FAKHREDDIN JAMALI*,*

Faculty of Pharmacy, University of Tehran (Iran)

(Received August 5th, 1982)

Ethyl biscoumacetate (EBA) is a coumarinic oral anticoagulant which differs from other drugs of its group by having a faster onset of action [1, 2]. For determination of EBA in biological fluids, only a spectrophotometric method has been reported [3]. In pharmaceutical samples, however, a high-performance liquid chromatographic (HPLC) method has been used to measure this drug [4]. In the process of studying the interaction of dipyrone with EBA [5] a rapid, sensitive and specific HPLC method suitable for measuring micro-quantities of EBA in human plasma was developed, which is reported below.

EXPERIMENTAL

Apparatus and conditions

The HPLC system consisted of a Model 224 U instrument (Waters Assoc., Milford, MA, U.S.A.) equipped with a dual-channel fixed-wavelength (254 and 280 nm) UV detector and a 30 cm × 3.9 mm I.D. column with a 10-μm (spherical) particle size (μBondapak C_{18}, Waters Assoc.). The mobile phase was methanol—water—acetic acid (56:40:4) which was pumped at a flow-rate of 1 ml/min with an initial pressure of 7 MPa.

Chemicals

All organic solvents were of analytical grade and the distilled water was deionized. Carbamazepine was used as internal standard (IS) which was extracted from tablets (Tegretol, Ciba Geigy, Iran) with benzene and recrystallized in absolute ethanol—benzene (70:30). The purity of IS and EBA (Ciba Geigy) was confirmed by the official methods [6, 7].

*Present address: Faculty of Pharmacy and Pharmaceutical Sciences, The University of Alberta, Edmonton, Alberta, T6G 2N8, Canada.

0378-4347/83/0000—0000/$03.00

Standard solutions

A stock solution of 40 mg EBA in 50 ml methanol was prepared and further diluted with water to provide four series of solutions containing 2, 4, 20, 40, 80, 120, 200, 400 and 800 μg in 3 ml of water. In 25-ml PTFE-insert screw-cap glass tubes, 1 ml blank plasma, 1 ml 0.1 *M* hydrochloric acid, 1 ml IS (40 μg/ml in methanol) and 10 ml benzene were added to the standard solutions. Tubes were shaken mechanically for 30 min. A blank solution without EBA and IS was also prepared. The benzene layer was removed and transferred into 15-ml centrifuge tubes, evaporated under vacuum and the dried residue was dissolved in 0.25 ml methanol. Aliquots of 25 μl of the latter solutions were injected into the HPLC system. The final amounts of EBA in 25 μl were 0.05, 0.10, 0.50, 1.00, 2.00, 3.00, 5.00, 10.00 and 20.00 μg, respectively, with a constant amount of 4.00 μg IS in each injected sample. Four series of standard solutions were prepared. The 280-nm peak height ratio (EBA/IS) was calculated and a standard curve was prepared by plotting the ratios versus amount of EBA. Statistical parameters were computed using a programmable calculator (Model 97, Hewlett-Packard, Corvallis, OR, U.S.A.).

Recovery

To determine the efficiency of the extraction, two series of solutions similar to those of the standard solutions were prepared but EBA (in methanol) was added to the separated benzene layer after the extraction.

Subjects

Volunteers were four healthy male students with average age and body weight of 26.5 years and 66.0 kg, respectively. They took single tablets of 300 mg EBA with 250 ml water after an overnight fast and at least 1.5 h before breakfast. Venous blood samples were taken from forearms by heparinized disposable syringes at 0, 0.5, 1, 1.5, 2.5, 3.5, 5, 7 and 9 h post-dosing. Samples were centrifuged and plasma portions were kept frozen until the time of analysis. The contents of 0.1—2.0 ml plasma were determined using the same procedure described in the *Standard solutions* section. The biological half-lives of EBA were estimated from slopes of plasma drug concentration–time curves using the last four points of the terminal phase.

RESULTS AND DISCUSSION

Fig. 1 depicts chromatograms from plasma before and 1 h after administration of a single oral dose of 300 mg EBA to a subject. No interfering peaks were noted from blank plasma. Peaks representing EBA and carbamazepine appeared 14.0 and 16.5 min, respectively, after injection into the chromatograph. The selectivity of the assay was assured by routine examination of UV absorbance ratios at 280/254 nm. Linearity and extraction recovery specifications are shown in Table I. For the pooled data the best-fit line through the points was described by $y = 0.1827x - 0.0013$ with a correlation coefficient of 0.999, indicating an excellent linear relation between the peak height ratio and the amount of injected EBA. Coefficients of variation (C.V.) varied from 2 to

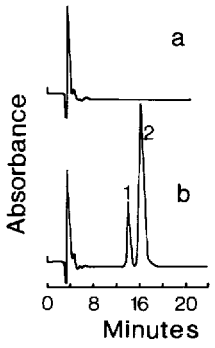

Fig. 1. Chromatograms of (a) blank plasma and (b) plasma of a subject 1 h after administration of 300 mg EBA. Peaks: 1 = EBA, 2 = carbamazepine (IS).

TABLE I

PEAK HEIGHT RATIOS (EBA/INTERNAL STANDARD) OBSERVED AT 280 nm FOR STANDARD SOLUTIONS (EXTRACTED) AND THEIR RECOVERY COMPARED WITH UNEXTRACTED SOLUTIONS

Amount added (μg)	Extracted*		Unextracted**	Recovered (%)
	Mean	C.V. (%)		
0.05	0.0077	24	0.0081	
			0.0080	95.6
0.10	0.0176	8	0.0187	
			0.0193	92.7
0.50	0.0879	5	0.0920	
			0.0904	96.4
1.00	0.1816	4	0.1882	
			0.1956	94.6
2.00	0.3609	4	0.3701	
			0.3668	97.9
3.00	0.5489	5	0.5681	
			0.5712	96.4
5.00	0.9187	4	0.9308	
			0.9453	97.9
10.00	1.8210	4	1.9250	
			1.9830	94.0
20.00	3.6535	2	3.8150	
			3.7500	96.6
Mean				95.7

*The best-fit line through the data is described by $y = 0.1827x - 0.0013$ which was used as standard curve (regression coefficient = 0.999). Mean value is the mean of four determinations.

**The best-fit line through the data is described by $y = 0.1902x - 0.0002$ (regression coefficient = 0.999).

8% within the examined range except for the solutions containing 0.05 μg per injection which was 24%. Therefore, the acceptable range was set as 0.10—20.00 μg. To measure plasma EBA concentrations below 0.1 μg/ml, therefore, larger plasma volume samples were required.

An average of 95.7% (standard deviation 1.7) was found to be extractable using this method (Table I).

Plasma EBA concentrations after administration of single oral doses of 300 mg are shown in Table II. Maximum plasma EBA concentrations (C_{max}) ranged from 10.21 to 14.62 μg/ml and were attained (T_{max}) 1.0—1.5 h post-dosing. In two subjects, 9 h after ingestion, plasma drug concentrations declined below the acceptable range (0.03 and 0.08 μg/ml in R.M. and M.J., respectively). Utilization of larger volumes of plasma and/or injection of larger volumes of extracts into the chromatograph could bring the levels within the desirable range. However, no attempts were made to increase the precision of these two samples.

TABLE II

PLASMA EBA CONCENTRATIONS (μg/ml) AND HALF-LIVES OF TERMINAL PHASES ($t_{½}$) AFTER ADMINISTRATION OF SINGLE ORAL DOSES OF 300 mg TO HEALTHY SUBJECTS

Subject	Hours								$t_{½}$* (h)
	0.5	1.0	1.5	2.5	3.5	5	7	9	
M.J.	—	2.29	10.21	10.01	7.91	2.66	0.44	0.08	0.82
R.M.	1.24	10.56	8.04	2.88	1.72	0.57	0.28	0.03	1.02
G.A.	3.32	4.11	11.62	10.10	7.24	5.62	2.08	0.79	1.66
R.A.	5.83	10.58	12.44	11.84	6.04	3.75	1.41	0.77	1.79

*Calculated from the slope of the best-fit line through the last four points.

Following oral administration of bolus 1500 mg and then daily maintenance doses of 300 mg EBA to five subjects, using a spectrophotometric method, Brodie et al. [3] were unable to quantitate the drug beyond 8 h post-bolus dose and 6 h post-maintenance dose. Therefore, they concluded that EBA does not cumulate in plasma during chronic therapy which agrees with our observation. The observed half-lives of the post-absorption phase of the plasma EBA concentration versus time curves ($t_{½}$) calculated from lines best-fitted through the terminal four points of each curve varied from 0.82 to 1.79 h with a mean of 1.36 h and standard deviation of 0.421 (Table II). This drug is usually administered in one or two divided daily doses [5, 8]. Dosing intervals of 8 and 12 h correspond to 6.7-h and 13.4-h half-lives observed after 300-mg doses, respectively. Therefore, no appreciable accumulation of EBA is expected during chronic therapy.

The average C_{max} reported by Brodie et al. [3] was 25 μg/ml after ingestion of 300-mg daily doses. No measure of deviation from this average was reported. Nevertheless, this value is substantially higher than the range observed by us. Since no significant accumulation is expected after repeated doses of EBA,

this discrepancy may be attributed to the nonspecificity of the spectrophotometric method [9].

It is worthy of mentioning that this method was also applied to measure urinary excretion of EBA. Only traces of the unchanged drug were found in urine. However, three other peaks with retention times of 5.8, 10.6 and 15.4 min and with respective UV absorbance ratios (280/254 nm) of 4.57, 0.30 and 1.70 were consistently detectable in urine of EBA-administered subjects (Fig. 2). These peaks, presumably, represent metabolites or degradation products [4] of EBA (absorbance ratio 2.5).

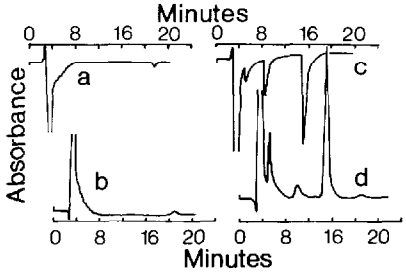

Fig. 2. Chromatograms of blank urine (a and b) and urine of a subject 2 h after administration of 300 mg EBA (c and d). Upper chromatograms represent absorbance at 254 nm while lower ones are observed at 280 nm.

ACKNOWLEDGEMENT

Supported by grant 400-3-57/2, Ministry of Culture and Advanced Education, Iran.

REFERENCES

1 M. Weiner, G. Simon, J.J. Burns, J.M. Steele and B.B. Brodie, Amer. J. Med., 4 (1953) 689.
2 J.B. Vander Veer, E.H. Funk, Jr., F.R. Boyer and E.A. Keller, Amer. J. Med., 4 (1953) 694.
3 B.B. Brodie, M. Weiner, J.J. Burns, G. Simson and E.K. Yale, J. Pharmacol. Exp. Ther., 106 (1952) 453.
4 R. Vanhaelen-Fastré and M. Vanhaelen, J. Chromatogr., 129 (1976) 397.
5 S.R. Mehvar and F. Jamali, Int. J. Pharm., 7 (1981) 293.
6 General Medical Council, British Pharmacopoeia, The Pharmaceutical Press, London, 1958, p. 264.
7 General Medical Council, British Pharmacopoeia, The University Press, Cambridge, 1980, p. 78.
8 W. Modell, Drugs of Choice, C.V. Mosby Co., St. Louis, MO, 1980, p. 632.
9 J.G. Kelley and K. O'Malley, Clin. Pharmacokin., 4 (1979) 1.