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Determination of ethyl biscoumacetate and its metabolite 7-hydroxyethyl biscoumacetate in human serum by high-performance liquid chromatography and mass spectrometry

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ABSTRACT

An efficient reversed-phase high-performance liquid chromatographic method has been developed for the determination of ethyl biscoumacetate (EBA) and its metabolite in human serum, using the μBondapak C_{18} column and methanol–water–phosphoric acid (56:46.8:0.2, v/v/v) as the mobile phase. This method permitted the determination of both EBA and a metabolite in human serum. The latter has been mentioned by other authors only in urine samples, where significant concentrations were found. Identification of the metabolite as 7-hydroxyethyl biscoumacetate was based on its chromatographic separation, followed by isolation from the eluate and direct mass spectrometric identification. It has been found that the higher EBA concentrations in human serum described by Brodie *et al.* [*J. Pharmacol. Exp. Ther.*, 106 (1952) 453] were caused by the insufficient resolving power of the spectrophotometric method used, leading to overlapping of the UV spectra of the parent drug and its metabolite.

INTRODUCTION

Ethyl biscoumacetate (EBA) is an anticoagulant with rapid onset of therapeutic effect, which rapidly disappears after discontinuing administration of the drug [1–3]. It has been found that EBA is almost completely metabolized in the body, but the chemical composition of the metabolite is very different in man, rabbits, rats and dogs [4]. The principal metabolite in human urine has been identified as 7-hydroxyethyl biscoumacetate (7-HEBA) [5]. It has been demonstrated experimentally that this metabolite enters the urine through a circuitous route involving biliary secretion [5]. These results suggest that 7-HEBA is formed in the liver and then secreted directly into the bile. Following biliary excretion into the intestine, 7-HEBA is absorbed into the bloodstream and eventually excreted in the urine. These studies have also demonstrated that 7-HEBA does not affect the prothrombin activity of plasma in mice. It should be pointed out that 7-HEBA has not yet been found in human blood. Various methods have been employed to monitor the EBA concentration in biological materials [1,4,6,7], but no evidence has been found for the occurrence of a metabolite in human plasma.

This paper describes the results of a study to demonstrate the presence of 7-HEBA in human blood, to verify the transport of this metabolite as suggested in ref. 5. High-performance liquid chromatography (HPLC) was employed to sep-

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arate 7-HEBA from the parent drug EBA in human serum and the metabolite was identified by UV and mass spectrometry (MS).

EXPERIMENTAL

Materials

Pelentan tablets, 300 mg of ethyl biscoumacetate substance, and the internal standard (I.S.; diazepam, DZM) were donated by Spofa (Prague, Czechoslovakia). Methanol was of HPLC grade (from E. Merck, Darmstadt, Germany). Chloroform of NMR grade (Merck) was employed for the extraction. All the other solvents and chemicals were of analytical reagent grade.

Subjects

A single tablet of Pelentan (300 mg of EBA) was administered to ten healthy male and female volunteers with an average age and body weight of 38.5 years and 69.8 kg, respectively, with a glass (300 ml) of water after overnight fasting and at least 2 h before breakfast. Venous blood samples were taken from the forearm using a syringe at 0, 0.33, 0.67, 1.0, 1.5, 2, 3, 4, 5, 6 and 8 h after administration. The samples were centrifuged for 10 min at 3000 *g* and serum portions were stored frozen at −20°C until the time of analysis.

Extraction procedure

A 100-μl aliquot of internal standard solution of DZM (200 μg/ml in methanol) and 100 μl of 1 *M* hydrochloric acid were added to 1 ml of serum. The samples were then extracted with 5 ml of chloroform by shaking for 5 min. After centrifugation (3000 *g* for 5 min), the organic layer was carefully transferred to another tube and evaporated to dryness at 40°C under reduced pressure. The residue was completely dissolved in 100 μl of 0.1 *M* sodium hydroxide and a 20-μl aliquot of this solution was injected into the chromatograph. This extraction procedure was also employed for preparation of a sample from urine.

For MS analysis, the samples were extracted from the mobile phase into the chloroform immediately after leaving the detector. The volume of the mobile phase related to the peak width at half-height of appropriate compound chromatographed was collected for this purpose. The chloroform extracts were combined and evaporated to a residue of 100 μl. Aliquots of 1–5 μl were injected into the mass spectrometer.

Chromatographic conditions

The PU 4100 liquid chromatograph was equipped with a PU 4021 multi-channel detector and a 30 cm × 3.9 mm I.D. column with a spherical particle size of 5 μm (μBondapak C_{18}, Waters Assoc.). The chromatograph was fitted with a 7125 Rheodyne injector and a 20-μl loop. The mobile phase was methanol–water–phosphoric acid (53:46.8:0.2, v/v/v), which was pumped at a flow-rate of 1 ml/min. The column was maintained at room temperature for the separation procedure.

Mass spectrometry

MS was carried out on an Incos 50 mass spectrometer (Finnigan MAT). Incos 50 software (version 9.00) was used for data acquisition and processing. Typical operating conditions: full-scan mode, 35–500 a.m.u. in 0.6 s, electron ionization at 100 eV, ion source temperature 150°C. Samples were evaporated by direct exposure to the probe at a heating rate of 10 mA/s.

Calibration curves

Calibration curve ($n = 8$) obtained from the peak area ratio for EBA/DZM in a range of standard concentrations yielded the linear regression equation $y = 0.6724 + 0.0213x$ and a correlation coefficient of $r = 0.997$.

Recovery

The EBA recovery was estimated by the analysis of standard solutions containing known amounts of EBA. The mean recovery was 95.4 ± 1.7% ($n = 10$).

Detection limit

The minimum quantifiable concentration was 0.1 μg of EBA per 1 ml of serum with a coefficient of variation of 11.8%.

Precision and accuracy

The within-day and day-to-day coefficients of

variation (concentration = 5 μg/ml) were 2.3% (n = 8) and 2.8% (n = 12), respectively. The mean intra- and inter-assay accuracy at EBA concentrations of 0.5–20 μg/ml were 103 ± 8 and 108 ± 7%, respectively.

Selectivity

No interference was observed from endogenous substances or as a result of contamination from tubes or containers.

RESULTS

In contrast to other authors [4,5], who did not mention any significant concentrations of metabolites in human plasma after administration of EBA, we found that an additional compound is present along with the parent drug in the samples prepared as described in the Experimental section. The chromatographic retention characteristics of this compound (designated A) are identical to those for a species found in human urine after administration of EBA (see Fig. 1). The identification of compound A was based on its chromatographic separation, followed by isolation from the eluate and direct MS determination.

The MS spectra of the parent drug and species A are given in Fig. 2A and B, respectively. The molecular ion of A is 16 m/z higher, corresponding to the addition of one atom of oxygen to the EBA molecule, *i.e.* to the formation of a hydroxy derivative.

The HPLC method was sufficiently sensitive to simultaneously follow the kinetics of EBA and its metabolite 7-HEBA in the serum of patients to whom a single oral dose of 300 mg of EBA was administered. For illustration, Fig. 3 depicts the time dependence of the mean concentration curves (n = 10) for EBA and 7-HEBA.

As pure 7-HEBA was not available, both species were quantified on the basis of the EBA standard. The very similar chemical structure and thus extractability suggest that the extraction and UV absorption coefficients will be very similar with a deviation in the range ± 10%.

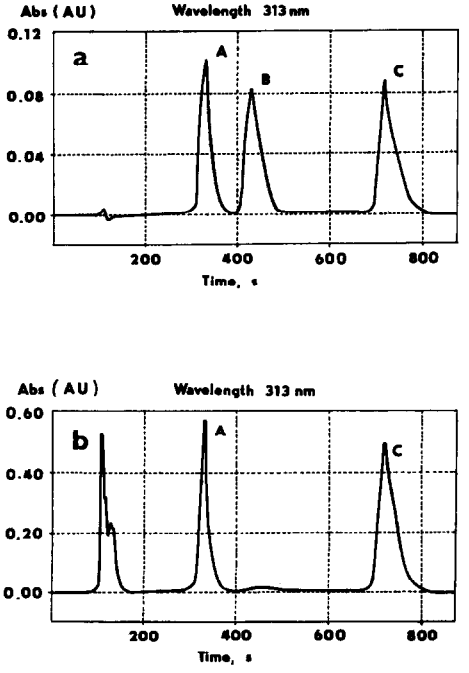

Fig. 1. HPLC of urine (a) and serum (b) of a subject 3.5 h after the administration of 300 mg of EBA. Peaks: A = 7-HEBA, 5.26 min; B = EBA, 7.11 min; C = DZM, 12.06 min.

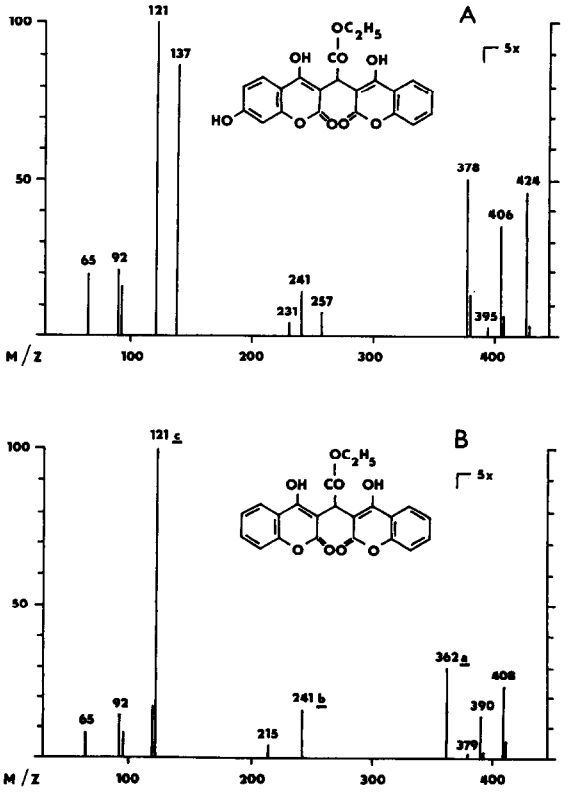

Fig. 2. Electron impact (100 eV) mass spectra of 7-HEBA (A) and EBA (B).

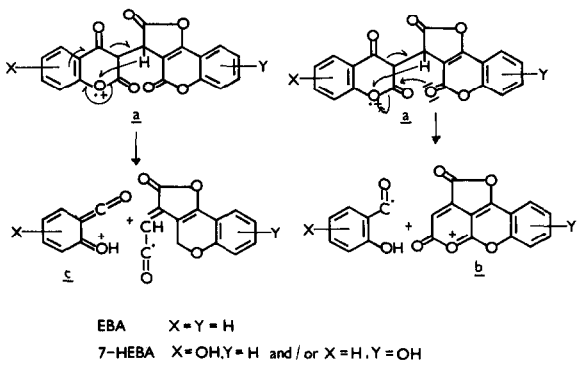

Fig. 3. Proposed scheme of EBA and 7-HEBA mass fragmentation.

DISCUSSION

Ethyl biscoumacetate and its metabolite yielded relatively intense molecular ions at *m/z* 408 and 424, respectively. The main fragments in the mass spectrum of EBA (Fig. 2B) are *m/z* 362 (a), *m/z* 241 (b) and *m/z* 121 (c). Loss of ethanol from the ionized molecule yields ions a. Ions b are probably formed from ions a through loss of a neutral fragment, $C_7H_5O_5$. Ions c can be produced from ions a through loss of neutral fragment $C_{13}H_5O_5$ or from ionized molecules through cleavage of the lactone connected with hydrogen transfer to oxygen-1. The mechanism has been shown to be operative for simpler 4-hydroxycoumarin derivatives [8]. In the mass spectrum of 7-HEBA (Fig. 2A), ions a are shifted from *m/z* 362 to *m/z* 378. Ions b and c are shifted only slightly as a consequence of the presence of an additional oxygen in one of the two coumarin moieties of EBA. Metabolic introduction of one oxygen atom into the EBA molecule does not change the basic fragmentation pattern. It thus follows that the hydroxyl group is probably present on the benzene ring of the coumarin part of the EBA molecule. The proposed fragmentation scheme is depicted in Fig. 3.

Burns *et al.* [5] have stated that the main metabolite of EBA in human urine is a hydroxyl derivative of the parent drug. The OH group is located on one of the benzene rings in position 7, as demonstrated by Hais and Procházka [9] and Fučík *et al.* [10]. The identical retention volumes of species A in our serum samples and of species A in the urine samples indicate that the two compounds are most probably identical. Arman and Jamali [7] also employed the HPLC technique for quantitative determination of EBA and also mentioned the presence of other species, presumably metabolites only in urine and not in the serum samples. It may be that the metabolite was not identified in the serum as a consequence of overlapping of the metabolite peak with the peak of the internal standard, as follows from comparison of the I.S. retention time in the serum sample with that of a species designated by Arman and Jamali [7] as a probable metabolite in the urine samples.

The systematically higher values for EBA in the serum samples described in the fundamental pioneering works of Brodie *et al.* [1] and Burns *et al.* [5], in contrast to the results of Arman and Jamali [7] and the results described here, are probably a consequence of superposition of the UV spectral bands of EBA and 7-HEBA. The fact that the spectral characteristics *per se* cannot usually differentiate the parent drug from its metabolite unless used in combination with an extraction and/or chromatographic procedure was mentioned in refs. 11 and 12.

Finally, it is evident from our data depicted in Fig. 4 that EBA is eliminated from the body more rapidly than was previously supposed.

CONCLUSIONS

(1) 7-HEBA has been isolated by the HPLC technique from human serum as the main metabolite of EBA. The identification was based on the results of mass spectroscopic analyses.

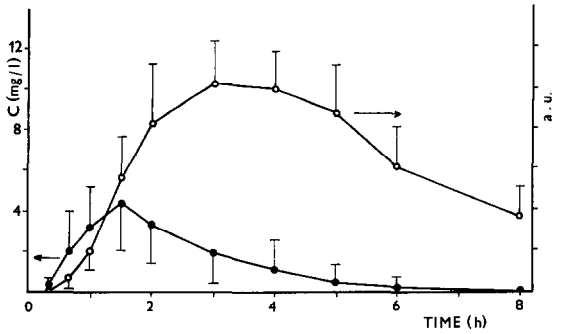

Fig. 4. Time course of mean (n = 10) concentration (C) curves for EBA (●) and 7-HEBA (○).

(2) The discrepancies between earlier and more recently published serum EBA concentrations were probably a consequence of insufficient resolving power of the spectrophotometric method originally used for EBA determination in biological fluids.

REFERENCES

1 B. B. Brodie, M. Weiner, J. J. Burns, G. Simson and E. K. Jale, *J. Pharmacol. Exp. Ther.*, 106 (1952) 453.
2 M. Weiner, G. Simson, J. J. Burns, J. M. Steele and B. B. Brodie, *Am. J. Med.*, 4 (1953) 689.
3 J. B. Van der Veer, E. H. Funk, Jr., F. R. Boier and E. A. Keller, *Am. J. Med.*, 4 (1953) 694.
4 I. M. Hais, B. Kakáč and L. Morávek, *Chem. Listy*, 46 (1950) 140.
5 J. J. Burns, M. Weiner, G. Simson and B. B. Brodie, *J. Pharmacol. Exp. Ther.*, 108 (1952) 33.
6 R. Vanhaelen-Fastre and M. Vanhaelen, *J. Chromatogr.*, 129 (1976) 397.
7 M. Arman and F. Jamali, *J. Chromatogr.*, 272 (1983) 406.
8 Q. N. Porter and J. Baldas, *Mass Spectrometry of Heterocyclic Compounds*, Wiley, New York, 1971, pp. 155–161.
9 I. M. Hais and Z. Procházka, *Thrombosis and Metabolism, 1st International Conference, Basel, 1954*, B. Schnabe, Basel, 1955.
10 K. Fučík, I. M. Hais, B. Kakáč, E. Knobloch and Z. Procházka, *Csl. Fysiol.*, 1 (1952) 277.
11 J. A. F. de Silva, *J. Chromatogr.*, 340 (1985) 14.
12 J. G. Kelly and K. O. Malley, *Clin. Pharmacokin.*, 4 (1979) 1.