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Short Communication

Reversed-phase liquid chromatographic determination of Eterobarb and its metabolites in plasma

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Introduction

Infantile epilepsy is a disease of social relevance, it has an incidence of 6–8/1000, and more than 50% of all epileptic crises occur with juveniles. Barbiturates have been used in therapy as anticonvulsants for more than 60 years and have up to now been a main point of anticomitial therapy, being effective in all epilepsy types with the exception of falling sickness (absences). Eterobarb (dimethoxymethylphenobarbital, DMMP; Fig. 1a) is an experimental anticonvulsant which has been reported to be effective in patients with generalized tonic-clonic and partial complex seizures which are resistant to standard anticonvulsant therapy. In addition, it is said to produce very little sedation compared with equivalent doses of phenobarbital [1–3]. Studies in animals and man have shown it to be rapidly metabolized to the monomethoxy derivative (MMMP; Fig. 1b) and to phenobarbital (Fig. 1c) [4–8].

Assay methods for DMMP and its metabolites previously extracted from acidified plasma involving descending [5] and ascending [9] thin-layer chromatography (TLC), and by the countercurrent distribution technique [5], have been described.

DMMP and its metabolites can also be measured by gas–liquid chromatography (GLC) following extraction from serum, without prior derivatization [8], or after methylation with trimethylphenylammonium or diazomethane [4]. Gas chromatography–mass spectrometry (GC–MS) also has been employed to monitor the metabolism of DMMP [10, 11].

The above techniques are relatively complex and need long preparation times, and even then are not suitable for routine use.

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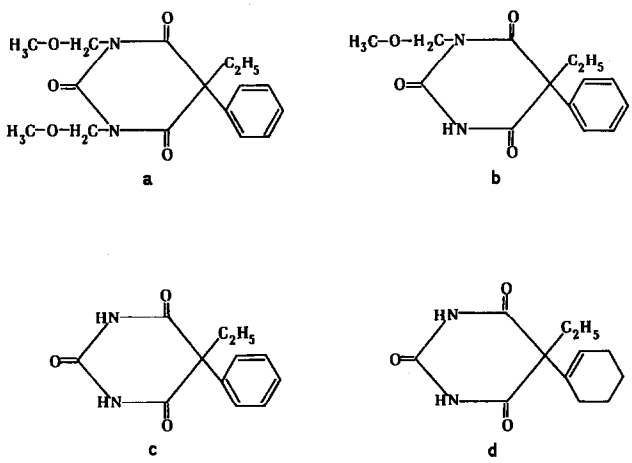

Figure 1
Structures of (a) Eterobarb (dimethoxymethylphenobarbital, DMMP); (b) monomethoxymethylphenobarbital (MMMP); (c) phenobarbital; and (d) cyclobarbital.

On the other hand, high-performance liquid chromatography (HPLC) has never been used for this specific problem, even though it is commonly used for the determination of other currently prescribed anticonvulsants [12].

The present work represents an attempt by the authors, who have previously studied hematic levels of barbiturates in children [13, 14], to develop a simple yet inexpensive HPLC method that can be easily adopted in drug control laboratories for the simultaneous determination of Eterobarb and its metabolites.

Experimental

Reagents and materials

DMMP and MMMP were kindly donated by Chiesi Farmaceutici (Parma, Italy), phenobarbital and cyclobarbital supplied by Merck (Darmstadt, FRG), sodium dihydrogen phosphate (Carlo Erba, Milan, Italy) were of analytical grade; water and acetonitrile (Carlo Erba, HPLC grade).

Instrumentation

The chromatograph was a Varian 8520 gradient system (Varian, Palo Alto, USA) equipped with Rheodyne injector (50 μl loop, Rheodyne, Berkeley, USA), and Pye Unicam P.U. 4021 multichannel detector (Pye Unicam, Cambridge, UK). Separation was achieved using a 250 × 4 mm, i.d., stainless steel column packed with 5 μm Hypersil ODS (Shandon, Runcorn, UK). The mobile phases were acetonitrile 0.01 M sodium dihydrogen phosphate (10:90, v/v% **A**) and acetonitrile (**B**) mixed in a gradient system (16% **B** for 6 min, followed by 5%/min linear increments from 16 to 35% **B**), delivered at a flow rate of 1.5 ml min^{-1}. The eluent was monitored spectrophotometrically at 212 nm. Quantitation was based on the use of the internal standard method (cyclobarbital). The peak area evaluation was obtained by a 3390A integrator (Hewlett–Packard, Palo Alto, USA).

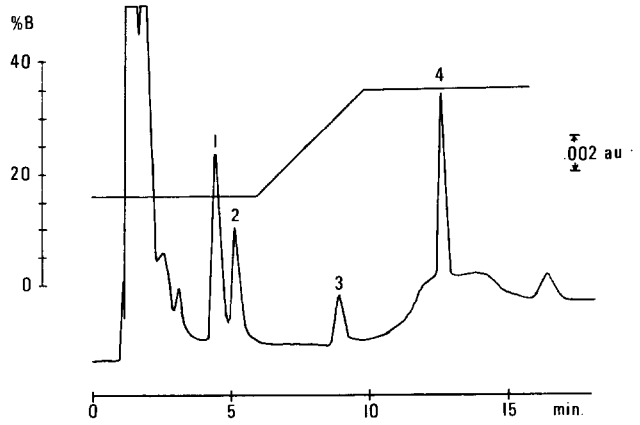

Figure 2
Chromatogram illustrating the separation in plasma of (1) phenobarbital, 4 μg ml^{-1}; (2) cyclobarbital, 4 μg ml^{-1}; (3) MMMP, 2 μg ml^{-1}; (4) DMMP, 4 μg ml^{-1}. Column: C_{18}-silica, 250 × 4 mm, i.d. Eluent: acetonitrile/0.01 M NaH_2PO_4 (10:90%, v/v), **A**; and acetonitrile, **B**. Gradient: 6 min 16% **B**, 5% linear increments from 16 to 35%. Flow rate: 1.5 ml min^{-1}. Detection wavelength: 212 nm. Sensitivity: 0.2 AUFS.

Calibration procedure

Weighed quantities of DMMP, MMMP and phenobarbital were added to 200 μl aliquots of blank plasma to yield concentrations of 9.1 μg ml^{-1} of DMMP, 4.2 μg ml^{-1} of MMMP and 6.9 μg ml^{-1} of phenobarbital, respectively, in 10-ml glass centrifuge tubes. To each tube was added 200 μl of a solution in acetonitrile containing 7.2 μg ml^{-1} of cyclobarbital (internal standard) whilst vortexing for 30 s. The tubes were then centrifuged for 10 min at 4000 rpm, and 50 μl aliquots of the supernatant were injected into the chromatograph. The measured peak areas corresponding to known quantities of each of the components and of the internal standard were used to calculate response factors. These were subsequently used to determine the concentrations of the test substances in samples to which known amounts of internal standard had been added.

Determination of total barbiturates in plasma

Volumes of 200 μl plasma from patients treated with DMMP were mixed with 200 μl of acetonitrile containing internal standard, vortexed and centrifuged as previously described, and 50 μl aliquots injected into the chromatograph.

Determination of free barbiturates

Volumes of 200 μl plasma from treated patients were ultrafiltered through "Centrifree" (TM, Amicon). After addition to the filtrate of 10 μl of acetonitrile solution containing 140 μg ml^{-1} of cyclobarbital and vortexing, 50 μl aliquots were injected into the chromatograph.

Results and Discussion

Many attempts at solid-phase extraction (Sep Pack C18, Waters, Milford, USA) have been made to purify the sample and consequently to improve the subsequent chromatographic separation, but the polarity of examined compounds renders difficult a

Table 1
Percentage recovery from plasma

Sample	Number of determinations	Weight added (ng)	Weight recovered (ng)	Percent recovery	RSD
Phenobarbital	10	172.5	164.7	95.5	±10
Cyclobarbital	10	180	170.6	94.8	±11
MMMP	10	105	100.2	95.4	±12
DMMP	10	227.5	216.6	95.2	±10

complete transfer from aqueous to organic stationary phase, and causes low and variable recoveries.

The proposed method enables the detection and quantitation of all of the investigated compounds at a concentration as low as 2 $\mu g\ ml^{-1}$, with a range of linearity from 2 to 100 $\mu g\ ml^{-1}$. Recovery of total barbiturates is 95% with a repeatability represented by a RSD of 10% (see Table 1).

Because of its rapid metabolic conversion to phenobarbital, levels of DMMP and MMMP in plasma of treated children reach zero, 10 h after administration.

The great difference in k' of phenobarbital, DMMP and MMMP necessitates the use of a gradient elution system, attempts at isocratic elution with various mobile phases failed to give satisfactory resolution. The choice of the internal standard was directed by the need to use a substance behaving as much as possible like the analytes, both in extraction and in chromatography, and readily available. The barbiturate (cyclobarbital) was selective even though not baseline separated from phenobarbital. However, when added to the sample, recoveries and linearity ranges were found to be similar to the examined barbiturates.

The use of small quantities of plasma were chosen for analysis in order to apply the method to young children. The method is at present in use for such purposes.

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