Journal of Chromatography, 118 (1976) 217–224

CHROM. 8809

RAPID AND SENSITIVE LIQUID CHROMATOGRAPHIC DETERMINATION OF CARBAMAZEPINE SUITABLE FOR USE IN MONITORING MULTIPLE-DRUG ANTICONVULSANT THERAPY

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(Received September 9th, 1975)

SUMMARY

A rapid, sensitive and selective method for the determination of carbamazepine and its major metabolite in plasma has been developed. Other commonly used anticonvulsants can be determined in the same procedure without interference.

After extraction with dichloromethane, the components are separated by high-pressure liquid chromatography without further clean-up or concentration on a column packed with small-particle silica gel. The mean recovery from plasma is 98.6% with a relative standard deviation of 1.6%. The detection limit for carbamazepine is approximately 2 ng/ml, requiring 1 ml of plasma.

INTRODUCTION

Carbamazepine (Tegretol®) is an iminostilbene derivative which is widely used in the treatment of convulsive disorders and trigeminal neuralgia. The quantitative determination of carbamazepine and its major metabolite, carbamazepine 10,11-epoxide which may also possess anticonvulsant properties, is important both for monitoring and optimizing blood levels of patients receiving therapeutic doses, as well as for the study of pharmacokinetic properties. As carbamazepine is often given in combination with other anticonvulsant drugs, such as phenobarbital and phenytoin, concomitant monitoring of plasma levels of the latter has also proved important for a rational therapy[1]. Therefore, an assay method for carbamazepine, in addition to being rapid, sensitive and selective, should be capable of producing accurate information on carbamazepine, its epoxide and other major anticonvulsant drugs.

A variety of spectrophotometric[2–4], thin-layer chromatographic[5–9] and gas chromatographic[10–18] methods is already available, but these do not fulfil the above requirements. The major disadvantage of the methods based on gas chromatography is that carbamazepine is not stable at the temperatures required to volatilize it which may result in decomposition into iminostilbene and other degradation products such as acridine derivatives[11,16,19]. It is therefore necessary to form more volatile carbamazepine derivatives, but this involves rather lengthy and tedious procedures.

In high-pressure liquid chromatography (HPLC), thermal decomposition rarely occurs and hence it seems to be the method of choice for separating non-volatile and/or thermally unstable components. Recently, two procedures for carbamazepine utilizing HPLC have been described[20,21], but both methods lack the capability to assay other anticonvulsants in the same chromatographic run. The present HPLC-method is able to monitor carbamazepine and its epoxide as well as other commonly used anticonvulsant drugs. and it also fulfills the requirements of speed, sensitivity and selectivity.

METHODS AND MATERIALS

Chemicals and reagents

Carbamazepine, carbamazepine 10,11-epoxide and 10,11-dihydrocarbamazepine were gifts from Ciba-Geigy (Basle, Switzerland). Pure carbamazepine was recrystallized from acetone. The product melted at 190–191°. Nitrazepam was obtained from Hoffmann-La Roche (Basle, Switzerland). All solvents and other chemicals were of analytical-reagent grade and obtained from E. Merck (Darmstadt, G.F.R.). Dichloromethane was redistilled prior to use.

Standard solutions

A standard solution of carbamazepine in distilled water was prepared at a concentration of 5 mg/100 ml. Carbamazepine was first dissolved in about 1 ml of ethanol (95%) before dilution with water. This solution was further diluted with water to produce solutions of the desired concentration.

Spiked plasma solutions (0.01–20 μg/ml) were prepared by transferring an aliquot (1 ml) of an aqueous carbamazepine solution to a 5-ml volumetric flask. This solution was brought to volume with citrated human plasma and the resulting solution mixed thoroughly.

Internal standard solutions, containing nitrazepam (3 μg/ml or 0.4 μg/ml) in water were also prepared by dissolving the required amount in about 1 ml of ethanol (95%) before dilution with water.

Apparatus

A Model 3500B liquid chromatograph (Chromatronix, Berkeley, Calif., U.S.A.) was used in this study. A Model SF 770 variable wavelength UV detector (Schoeffel), was operated at 250 nm. Injections were achieved with a high-pressure sample injection valve (Chromatronix, HPSV), fitted with a 50-μl sample loop.

The column, precision-bore stainless steel (25 cm × 2.1 mm I.D.), was packed with silica gel, specific surface area 400 m^2/g, $d_p = 10$ μm, (LiChrosorb SI 100; E. Merck) by a balanced-density slurry technique[22]. The slurry solvent consisted of tetrabromoethane–dioxane–chloroform (1:1:1). Prior to use, the tetrabromoethane was passed through a silica gel column (Kieselgel 0.05–0.2 mm reinst, E. Merck), to remove impurities. A 30-cm stainless-steel column of 9.0 mm I.D. was used as container for the slurry. The slurry (10%, w/w), degassed in a ultrasonic generator, was pumped into the chromatographic column at the highest possible flow-rate. Columns were washed by passing 200 ml of *n*-hexane and further equilibrated with the mobile phase. Analyses were performed using a mobile phase of 5% tetrahydro-

furan in dichloromethane at a flow-rate of 60 ml/h (68 kg/cm²) at room temperature (22–25°). The mobile phase was degassed ultrasonically before use.

Extraction procedures

Normal human plasma concentrations (0.5–20 μg/ml). To 0.5 ml of plasma in a 10-ml screw-capped centrifuge tube was added 0.5 ml of internal standard solution, containing 1.5 μg of nitrazepam. This mixture was extracted with 2.0 ml of dichloromethane by shaking vigorously for 30 sec. After separation of the layers, the aqueous phase was removed by aspiration and, without further purification, 50 μl of the dichloromethane phase were injected into the liquid chromatograph.

Subclinical plasma concentrations (0.01–0.5 μg/ml). An aliquot (1.0 ml) of the plasma sample was transferred to a conical centrifuge tube, 0.5 ml of internal standard solution, containing 0.2 μg of nitrazepam, and 6.0 ml of dichloromethane were added. The tubes were centrifuged for 2 min at 6000 *g* and the aqueous layer was removed by aspiration. An aliquot (5.0 ml) of the organic phase was transferred to a conical evaporation tube. The dichloromethane was evaporated at 40° in a stream of dry air. The residue was redissolved in 0.1 ml of mobile-phase solvent and 50 μl of this solution were injected into the liquid chromatograph.

Calibration graphs

Carbamazepine concentrations in plasma were calculated with the aid of calibration graphs. Spiked plasma samples were carried through the extraction procedures described, and the peak height ratio of carbamazepine to internal standard was plotted against the known plasma concentration of carbamazepine.

Recovery

Recoveries of carbamazepine at different concentrations were determined by extracting spiked plasma samples, after which nitrazepam was added as external standard. The peak height ratios of carbamazepine to external standard (R_1) were compared with the ratios (R_2) obtained by direct injection of the same amounts of carbamazepine and nitrazepam in dichloromethane.

$$\text{Recovery } (\%) = \frac{R_1}{R_2} \times 100$$

RESULTS AND DISCUSSION

Liquid–solid chromatography was chosen as separation method, because of the non-ionic character of carbamazepine. Since several studies[22–28] have shown that column efficiency in liquid chromatography can be improved by decreasing the particle size of the packing material, columns packed with small-particle silica gel were used. In order to pack these small particles in an efficient and reproducible manner, the so-called balanced-density slurry packing technique was used[22]. A stable suspension was prepared in a solvent with approximately the same density as the packing material, to minimize particle-size segregation during packing. Very efficient columns could be obtained in this manner. Columns tested with nitrobenzene as solute ($k' = 7.0$) in *n*-hexane (50% water saturated) as mobile phase at a flow-rate of

1 cm/sec exhibited an efficiency of 5000–10,000 theoretical plates per 25 cm. Column efficiency was not reduced even after analysis of more than 300 samples in about 6 months.

A simple binary solvent mixture, consisting of dichloromethane modified with 5% tetrahydrofuran proved to be useful. This mixture was choosen after testing solvents containing increasing amounts of tetrahydrofuran in dichloromethane. In Fig. 1 the capacity factor (k') of some commonly used anticonvulsant drugs is plotted *versus* the percentage of tetrahydrofuran in dichloromethane. It can be seen that addition of tetrahydrofuran to dichloromethane can influence retention time as well as column selectivity. Addition of 5% tetrahydrofuran appeared to be a good compromise between analysis time and resolution.

Fig. 2 shows a typical liquid chromatographic separation of a plasma extract, containing phenobarbital, phenytoin, nitrazepam (internal standard), carbamazepine and carbamazepine 10,11-epoxide. No interfering substances are extracted from

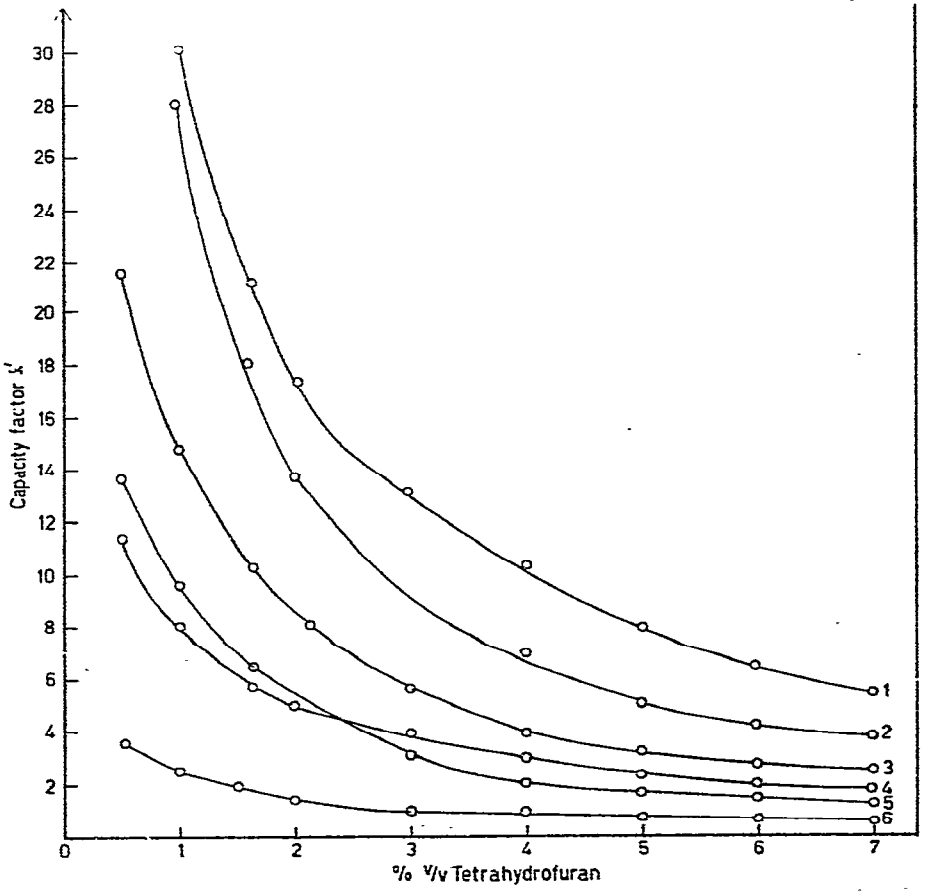

Fig. 1. The effect of the percentage tetrahydrofuran in dichloromethane on the capacity factor (k') of some anticonvulsant drugs. 1 = Carbamazepine; 2 = nitrazepam; 3 = phenytoin; 4 = ethosuximide; 5 = phenobarbital; 6 = methylphenobarbital.

plasma and analysis is achieved in less than 8 min. Carbamazepine 10,11-epoxide, the major metabolite of carbamazepine and having anti-epileptic properties in the rat[29], can also be assayed simultaneously by this procedure. However, the sensivity for this compound is lower, as has already been pointed out by Eichelbaum and Bertilsson[21], because it has no double bond in the 10,11-position (carbamazepine, $A^{1\%}_{1cm} = 403$ at 250 nm, and carbamazepine 10,11-epoxide, $A^{1\%}_{1cm} = 103$ at 250 nm in ethanol). Carbamazepine concentrations in plasma down to 10 ng/ml can be determined quantitatively. The lower limit for carbamazepine detection is about 2 ng/ml of plasma, using the procedure described under subclinical plasma concentrations.

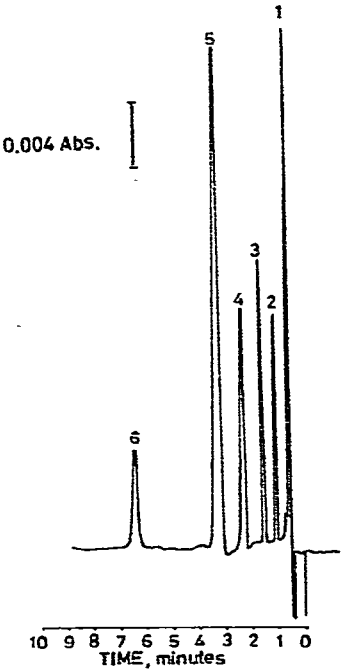

Fig. 2. Liquid chromatogram of a plasma extract. 1 = Solvent front; 2 = phenobarbital; 3 = phenytoin; 4 = nitrazepam; 5 = carbamazepine; 6 = carbamazepine 10,11-epoxyde. Mobile phase: 5% tetrahydrofuran in dichloromethane. Flow-rate: 60 ml/h at 68 kg/cm^2. Column: LiChrosorb SI 100, 10 μm, 25 cm × 2.1 mm I.D.

Extractions were performed with dichloromethane because carbamazepine is very rapidly extracted with this solvent. Extraction time is therefore not very critical.

The procedure described for concentrations ranging from 0.5–20 μg/ml, is useful for normal human plasma values.

Because no evaporation step is included, this method is very rapid and simple and thus especially suitable for routine analysis. 60–100 samples can be handled in one day using this procedure. In order to be able to measure nanogram amounts of carbamazepine in plasma, the previous procedure was slightly adapted, especially to study the pharmacokinetics of carbamazepine in man and animals.

Quantitation was performed by peak height measurement. This method has a good precision because the solute peaks are almost symmetrical.

Nitrazepam was used as an internal standard throughout this study because of its general availability; however, 10,11-dihydrocarbamazepine ($k' = 7$) can be used, if necessary, as an alternative, *e.g.* if nitrazepam is prescribed as medication.

Fig. 3 represents the calibration graph for the extraction procedure used for normal human plasma concentrations. The data are mean values of at least eight determinations. Relative standard deviations did not exceed $\pm$ 1.5%. It can be seen from this graph that the procedure is highly reproducible and has a good precision over a large concentration range. The efficiency of the extraction is shown in Fig. 4.

Recoveries were determined in the same concentration range by means of a method described by Breimer and Van Rossum[30] and appeared to be constant with a mean value of 98.6% and a relative standard deviation of 1.6% ($n = 40$). The proposed HPLC method offers the possibility to measure blood levels of several anti-epileptic drugs in the same chromatographic run and it has been applied successfully to the determination of blood levels of patients, receiving therapeutic doses of several anticonvulsive drugs.

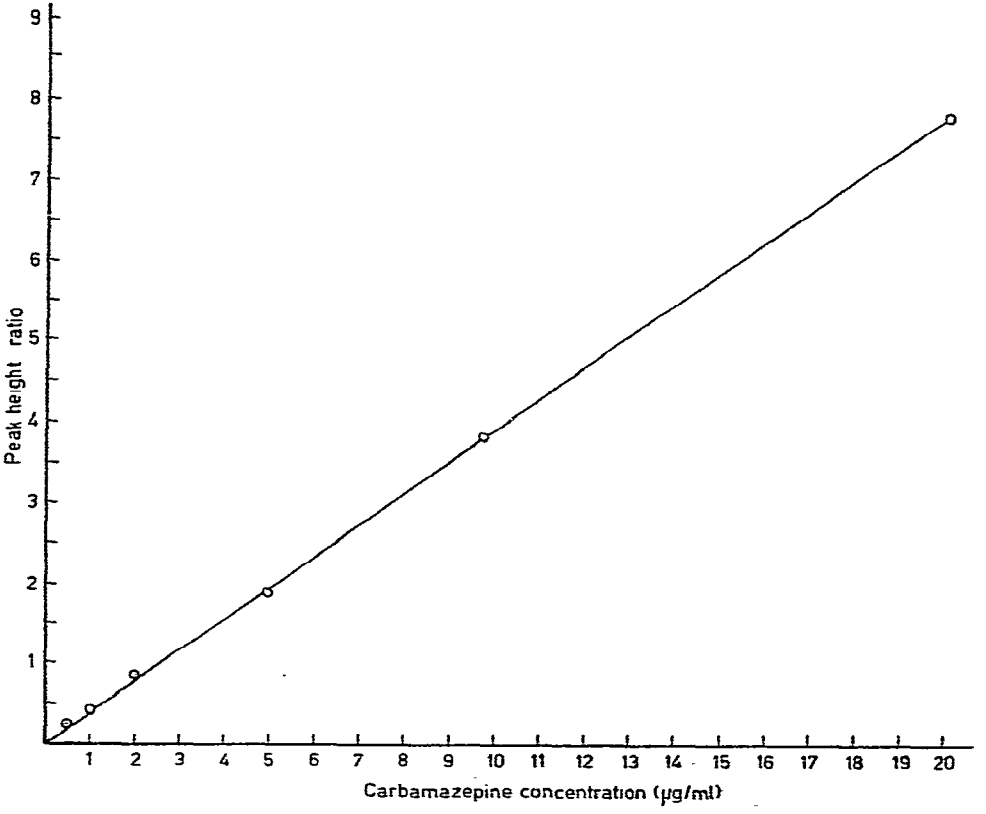

Fig. 3. Calibration graph for the determination of carbamazepine in plasma using nitrazepam as internal standard. Mean values of at least eight determinations. Coefficient of variation $\pm$ 1.5%.

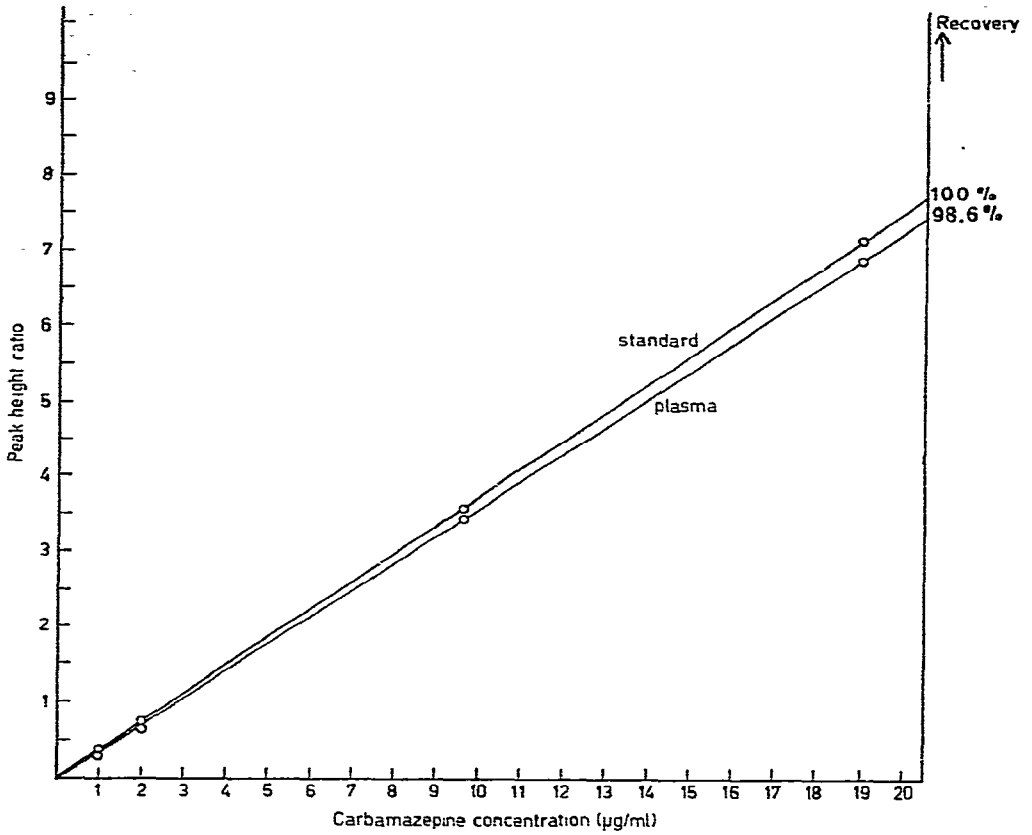

Fig. 4. Peak-height ratio of carbamazepine to nitrazepam as a function of known carbamazepine concentrations for the determination of recovery. For exact procedure, see text.

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