

SHORT COMMUNICATION

A REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR SIMULTANEOUS ESTIMATION OF MELATONIN, CARBAMAZEPINE EPOXIDE AND CARBAMAZEPINE SIMULTANEOUSLY IN SERUM

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Abstract : Monitoring of plasma antiepileptic drugs is useful for better clinical management in epileptic patients, particularly in children. Carbamazepine (CBZ) is one of the commonly prescribed anticonvulsants. The active metabolite of carbamazepine-carbamazepine-10-11 epoxide (CBZ-Epo) also exhibits anticonvulsant effect. The pineal hormone, melatonin exerts an anticonvulsant effect in experimental seizure models and recently has also been used in cases of childhood epilepsy. To facilitate the simultaneous plasma estimation of carbamazepine, carbamazepine epoxide, and melatonin, a new HPLC method was developed. Waters millennium 2010 chromatography manager with a 515 HPLC pump and Waters 24879 dual absorbance UV detector was used. A 25 microlitre of sample and standards were injected, and chromatographic separation was achieved by Merck C18 reverse phase column particle size 5 μ , 250 mm \times 4 mm. It was quantitated at UV light 210 nm. The retention times of melatonin, CBZ-Epo, and CBZ were 6.3 min, 7.5 min, and 13.9 min respectively. The Mobile Phase used was water: acetonitrile (70:30), pH 3.0 adjusted with orthophosphoric acid at the flow rate of 1 ml/min. The limits of detection of melatonin, carbamazepine epoxide, and carbamazepine were 800, 500, and 1300 pg respectively.

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INTRODUCTION

Evidence has accumulated about the involvement of reactive oxygen species (ROS) in epilepsy (1). The free radicals generated cause a cascade of neurochemical events leading to neurodegeneration. Ironically, though some antiepileptics like zonisamide inhibit free radical generation (2) some like carbamazepine (CBZ) are known to generate free radicals (3). Carbamazepine is one of the commonly prescribed anticonvulsants. The active metabolite of carbamazepine-carbamazepine-10-11 epoxide (CBZ-Epo) also exhibits anticonvulsant effect (4). In several studies the pineal hormone, melatonin which has the neuromodulatory effect, has shown anticonvulsant effect in different seizure models in rodents (5, 6, 7). It has recently been used in childhood epilepsies (8, 9). The anticonvulsant and the neuroprotective activity of melatonin have been suggested to be due to its antioxidant, anti-excitotoxic and free radical scavenging properties within the central nervous system (10). Advantageously, melatonin has been shown to antagonize the mutagenic effect of CBZ in some systems (11). A study in patients with intractable seizures showed decreased melatonin in those with temporal lobe epilepsy compared with controls. In the same study, an increase in melatonin and cortisol level in the 24 hour period following seizures, implicate the melatonin surge following seizures to be protective against repetitive seizures (12). In another study, it was demonstrated that in febrile as well as epileptic children, melatonin levels were significantly increased as compared to normal children (13). The estimation of melatonin along with the conventional antiepileptic drug

received by patients may be important in these patients.

High-performance liquid chromatography (HPLC) combined with a UV detector is the most common method for identification and quantification of antiepileptic drugs in biological fluids. Several HPLC methods have been presented for simultaneous determination of antiepileptic drugs in serum or plasma (14). However, no method was described for the simultaneous determination of melatonin, carbamazepine, and carbamazepine epoxide in human biological fluids. This is attributed to the extraction and reconstitution difficulties from biological fluids that are linked to their chemical properties. In this paper, we report a simple, sensitive and reliable reverse phase HPLC assay for the simultaneous determination of carbamazepine, carbamazepine epoxide, and melatonin in human serum using ultraviolet detection. This method has been successfully employed to monitor plasma serum levels of these drugs following oral administration in epileptic children.

METHODS

HPLC-Waters millennium 2010 chromatography manager with a 515 HPLC pump and Waters 24879 dual absorbance UV detector, USA were used. Reverse phase C18 column (150 mm × 4 mm; particle size 5 µm) was used. All solvents were of HPLC grade. All the reagents were used without further purification. Deionized water, purified by Milli Q system (Millipore, Milford, MA, USA), was used throughout the study.

Suitable quantities of melatonin, carbamazepine epoxide and carbamazepine in volumetric flask were weighed, and diluted with suitable quantities of acetonitrile to required concentrations. In one ml of serum sample, one ml of acetonitrile was added, shaken gently, centrifuged at 3 to 4 thousand RPM for 20 to 30 minutes. Then, the sample was decanted in HPLC vial directly and injected for quantification. A 25 microlitre of sample and standard were injected, and the contents of melatonin, carbamazepine epoxide and carbamazepine were calculated. Chromatographic separation was achieved by Merck C18 reverse phase column particle size 5 μ , 250 mm \times 4 mm. It was quantitated subsequently by exposure to UV light at 210 nm. The retention times of melatonin, CBZ epoxide, and CBZ were 6.3 min, 7.5 min, and 13.9 min respectively. The Mobile Phase consisted of Water: Acetonitrile (70:30) at pH 3.0 adjusted with orthophosphoric acid at the flow rate of 1 ml/min.

Assay validation

Samples were quantified using peak area of melatonin, carbamazepine, and carbamazepine epoxide. Standard calibration curves were constructed by spiked drug-free pooled human plasma with a known amount of melatonin, carbamazepine, and carbamazepine epoxide. These plasma standards were also used to determine the extraction recovery, within day and between-day precision ($n=5$) of the method. Limit of quantitation is based on the lowest concentration validated by the method.

RESULTS AND DISCUSSION

In the current study, we developed a

simple HPLC method for simultaneous determination of melatonin, carbamazepine, and carbamazepine epoxide in human serum. The solubility and polarity of the three analytes are vastly different, thus requiring different grades of polar solvents to extract and constitute. After extraction, the final component reconstituents were mixed to enable a single injection into the HPLC by the reverse phase method. In addition, peak purity of plasma samples as compared with standards can be monitored by this detection method.

Standard curves of melatonin, carbamazepine epoxide, and carbamazepine were obtained. Linear regression analysis of the curves described by plotting AUC (area under the curve) versus quantity injected indicated a linear fit of the data. The limits of detection of melatonin were about 800 pg; carbamazepine epoxide about 500 pg and carbamazepine about 1300 pg.

The described HPLC method is a new reverse phase high performance liquid chromatography method for extraction, separation and quantification of melatonin, carbamazepine epoxide and carbamazepine simultaneously in serum sample. The procedure employs a relatively simple liquid phase clean-up procedure for sample preparation. This method can be utilized in studies that require simultaneous estimation of serum melatonin, carbamazepine, and carbamazepine epoxide levels, for example, in patients of refractory, epilepsy, and can be a useful and a sensitive tool for therapeutic drug monitoring for clinical management in such epileptic patients.

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