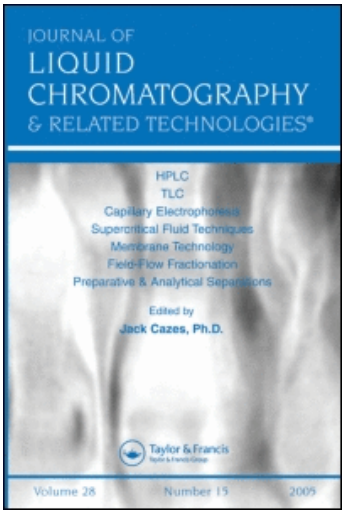

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A SENSITIVE METHOD FOR THE DETERMINATION OF BACLOFEN IN HUMAN CSF BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Baclofen (4-amino-3p-chlorophenylbutyric acid) is used clinically for the treatment of multiple sclerosis and other spastic conditions. The intrathecal route of administration is now prefered to the oral route. To optimize efficacy, the levels of the drug in CSF need to be monitored after intrathecal administration. In this paper, the authors describe a sensitive reverse phase high performance liquid chromatographic method for the determination of baclofen in human CSF. This assay employed cation exchange extraction, pre-column derivatization with PITC, and ultra-violet detection

(254 nm). The method was shown to be highly sensitive (5 to 10 ng/ml). This method was compared to a method using direct spectrophotometric detection of baclofen at 220 nm after direct application of CSF samples onto the chromatograph without prior extraction.

INTRODUCTION

Baclofen, 4 amino-3-p-chlorophenyl butyric acid is used in the treatment of multiple sclerosis and other spastic conditions for its antispasticity properties (1,2). Various authors (3-5, 6) have shown that intrathecal administration of this agent has considerable advantages over the oral route. However, there is considerable individual variation in the therapeutic levels required. In order to achieve optimum efficacy, drug levels need to be monitored in CSF after intrathecal administration.

Few methods are available for quantitation of this drug. A gas-liquid chromatographic method (7) and a mass fragmentographic method (8) have been described. More recently, high performance liquid chromatography with ultraviolet (9, 10) or fluorimetric detection (11) has been employed. A high performance liquid chromatographic method after precolumn extraction and derivatization with o-phtaldialdehyde (12) has also been described. In practice, a specific and sensitive method is required for pharmacokinetic studies since low doses (50-100 μg) are administered intrathecally.

We describe here a liquid chromatographic method (II) with ultraviolet (254 nm) detection, following precolumn derivatization with phenylisothiocyanate (PITC). This method was compared to a rapid reversed-phase HPLC method (I) with ultraviolet detection (220 nm).

MATERIALS

Chemicals and reagents. All solvents were of analytical reagent grade, and water was tridistilled. Chemicals were purchased from Merck, Darmstadt; Prolabo, Paris; Carloerba, Milan; and Pierce, Rockford. Baclofen powder was

obtained from Ciba-Geigy (Basle, Switzerland). A standard solution of 50 µg/ml prepared with the powder was diluted with water or CSF to produce concentrations ranging from 10 to 500 ng/200 µl.

Apparatus : HPLC. The HPLC system consisted of a Waters chromatograph equipped with a U6K injector, two Waters 510 solvent delivery systems, a M481 wavelength detector (220, 254 nm) and an automated gradient controller (Waters). A Waters M 990 photodiode array UV spectrophotometer was used to identify the samples. The temperature was controlled (38°C) with a column heater (Waters).

Sampling of CSF. CSF samples were collected (D11-D12) from a patient with multiple sclerosis. This patient had been implanted with a programmable drug pump (Medtronic) connected to a lumbar subarachnoid catheter (D9-D10). The patient received a continuous intrathecal infusion of baclofen (140 µg/day). The kinetics of baclofen in CSF were determined after shutting off the pump. Samples (0.5 ml) were collected at 0.5, 1, 2, 3, 4, 5, 7, 10, and 24 hours after stopping the infusion. The samples were immediately frozen.

METHODS

Two chromatographic methods were employed:

Method I. The HPLC column was a reverse phase uBondapak C18 column (30 cm x 0.39 cm). The CSF samples were injected directly without prior extraction. Baclofen was eluted isocratically at a flow rate of 1 ml/min with a mixture of methanol and 50 mM ammonium phosphate (20:80) adjusted to pH 6.22 with trifluoroacetic acid.

Method II. Samples were extracted using a Dowex 50X4-400 resin packed in a Bio-Rad column (0.7 x 10 cm). The analytical column was a "Pico-Tag" reverse phase column packed over 15 cm x 0.39 cm. 5-25 µl aliquots were injected into the chromatograph after dissolution in Na2HP04 (pH 7.4 adjusted

with 10% phosphoric acid). Separation was carried out on a gradient made up of two eluents: sodium acetate adjusted to pH 6.4 with glacial acetic acid, and 60% acetonitrile in water.

Preparation of the sample : Extraction and derivatization. We used a modification of the method described by Swahn et al. (8). To remove impurities that might interfere with the analysis, the Dowex was treated with an excess of aqueous ammonia, washed with water to neutrality, regenerated with an excess of 4 M HCl, and finally washed with water to neutral pH. The columns were then rinsed with 8 ml of water. 200 µl samples of CSF were allowed to flow through the resin which was then washed with 8 ml of water. The column was eluted with 2 ml of 10% ammonia, and fractions were collected in glass tubes. The samples were immediately frozen and lyophilized. After lyophilization, the residue was taken up in 1 ml of phosphate buffer (pH 7.4), and extracted twice with 1 ml of 1-n butanol. The butanol phases were evaporated, and the derivatization reagent was added. We used a modification of the method described by Bidlinmeyer et al. (13) for analysis of amino acids using pre-column derivatization. This method relies on formation of the phenylthiocarbamyl (PTC) derivatives of amino acids. Separation was carried out on a high-performance reversed-phase column. The standards and the drug residue were treated with 10-50 µl of a redrying solution consisting of ethanol-water-triethylamine (2:1:1). The samples were evaporated, and 20 µl of the derivatization reagent (ethanol-triethylamine-water-phenylisothiocyanate) were added to the residue. The reaction was complete after 20 minutes at ambient temperature. Excess reagents were then eliminated.

RESULTS AND DISCUSSION

A calibration curve was established using 200 µl of CSF. For each collection time, drug levels were quantified from the linear relationship between peak area versus concentration, obtained from samples of CSF spiked

with known quantities of baclofen. This was linear over a concentration range of 10 to 500 ng/200 µl.

Method I. In this method, the CSF was injected into the high performance liquid chromatograph without prior extraction of biological samples. Typical chromatograms of baclofen are shown in Figs 1 (1A, blank CSF sample ; 1B, a CSF sample after lumbar administration of baclofen ; 1C, a CSF sample spiked with a known amount of baclofen). No interfering susbtances were observed in the blank CSF. The retention time of baclofen using this method was 6.9 min. The identity of baclofen in the CSF samples was confirmed by comparing the ultraviolet absorption spectrum of the CSF samples with that of a baclofen standard (Fig. 3), using a Photodiode-Array.

Method II. In the second method, the extraction efficiency was 50% +/- 5%. Typical chromatograms of baclofen are shown in Figs 2 (2D, blank CSF sample ; 2E, a CSF sample spiked with a known amount of baclofen). No interfering substances were observed in the blank CSF. Under these HPLC conditions, the retention time of baclofen was 13.2 min. The detection limit in CSF was 5-10 ng/ml. No interference was observed from any endogenous CSF components.

There was a good correlation between the results obtained from the two methods (direct 220 nm UV, or PITC derivatization, cf. Fig. 4). Despite the rapidity of the direct UV method (no prior extraction, no derivatization), sensitivity was limited (50 to 100 ng/ml). For low concentrations (< 50 ng/ml) at high sensitivity, the chromatograms were not well resolved. In addition, since CSF was analyzed without purification, and since these patients are often treated with other drugs which may interfere with the determination of baclofen, we developed a more sensitive and specific HPLC method (5 to 10 ng/ml) after derivatization with PITC. However, this method is time consuming, and requires preliminary extraction, although it was found to be more sensitive (5 to 10 ng/ml) than other methods described in

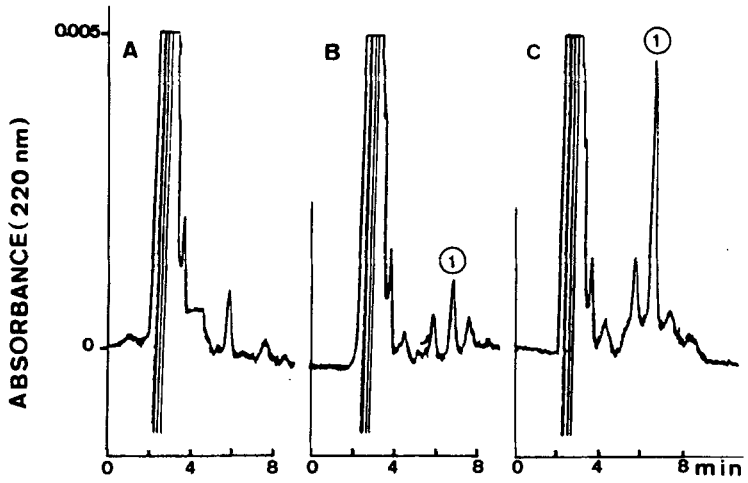

FIGURE 1: HPLC profiles (absorbance 220 nm) of (A) blank CSF, (B) patient CSF sample (560 ng/ml baclofen), (C) CSF sample spiked with 10 ng baclofen. 1 = baclofen.

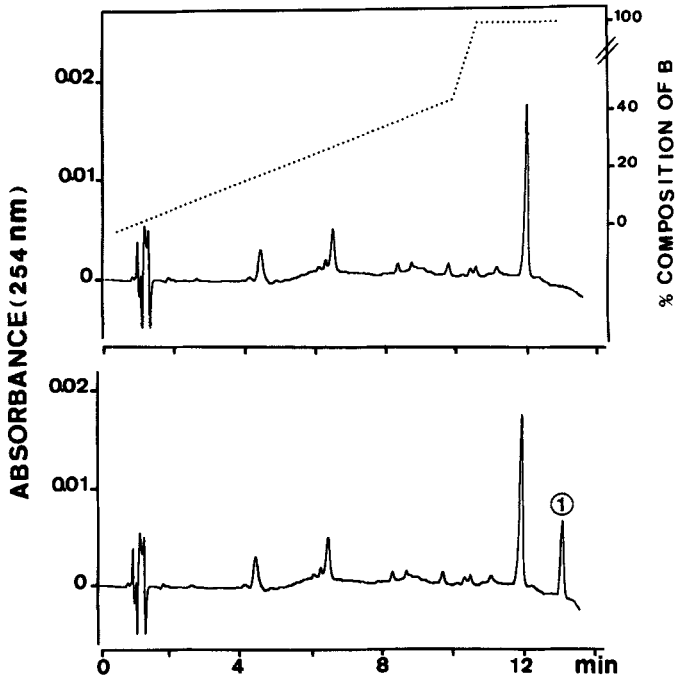

FIGURE 2: HPLC profiles (absorbance 254 nm) of (D) blank CSF, (E) CSF sample spiked with 10 ng baclofen. 1 = baclofen. The acetonitrile gradient used to elute baclofen is represented by the dashed line.

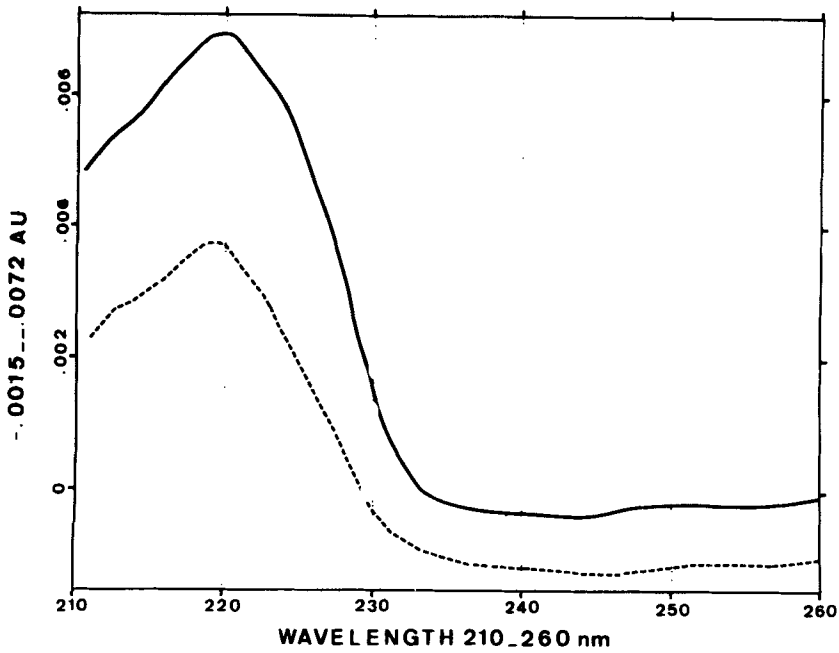

FIGURE 3: Ultra-violet spectrum (photodiode array Waters M 990) of baclofen : comparison of a baclofen standard (—) and patient's CSF (----).

the literature (6, 8, 10). Such methods are not sufficiently sensitive or accurate for assay of baclofen in CSF after intrathecal administration of low doses (100 µg). Although the mass fragmentographic method (7) had a similar sensitivity to our method, it is particularly time consuming. Derivatization with o-phthaldialdehyde (8) after precolumn extraction has been used for pharmacokinetic studies in human plasma and urine. The main drawback of this method was that the product is detected by fluorescence, and the authors did not specify the sensitivity.

In conclusion, both HPLC methods described here can be used to measure baclofen levels in CSF from patients receiving low doses of the drug via the intrathecal route. For low concentrations (< 50 to 100 ng/ml), the longer PITC method is required, while for higher concentrations (> 50 to 100 ng/ml) the direct UV method appears adequate.

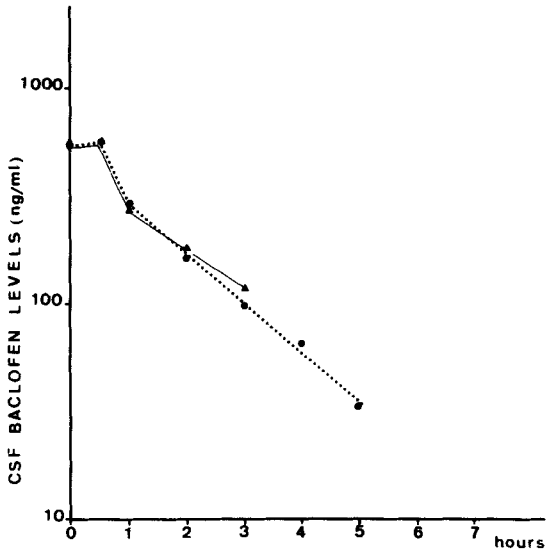

FIGURE 4: CSF baclofen concentrations after stopping the infusion (140 µg/day) : (—) Method I (220 nm) ; (•••••) Method II (254 nm).

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