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Note

Simultaneous measurement of chloramphenicol and chloramphenicol succinate by high-performance liquid chromatography

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Chloramphenicol (CAP) is a broad spectrum antibiotic which is currently experiencing a resurgence of use in pediatrics for the treatment of ampicillin resistant *Haemophilus influenzae*.

Several recent papers [1,2] have recommended that CAP serum concentrations be routinely monitored during treatment to assure therapeutic levels of the drug and to avoid concentration-dependent toxicity. Analysis of CAP has been reported utilizing microbiological [3], colorimetric [4], gas chromatographic [5], enzymatic [6] and high-performance liquid chromatographic (HPLC) [7,8] methods. The HPLC methods are reliable, easy to perform and, due to the small serum volume required, ideally suited for CAP quantitation in pediatric patients. However, these methods do not describe the simultaneous analysis of chloramphenicol succinate (CAPS) and CAP.

CAP is administered intravenously as its succinate ester which is biologically inactive and must be hydrolyzed in the body to free CAP. We, as well as others [9], have found no correlation between the dose of CAPS administered and blood levels of free CAP. The purpose of this paper is to describe an improved HPLC method for simultaneous quantitation of CAP and CAPS in serum, urine and cerebrospinal fluid (CSF).

MATERIALS AND METHODS

Chromatography

All assays were performed on a Perkin-Elmer model 601 high-performance liquid chromatograph equipped with a LC55 UV/VIS variable-wavelength detector and interfaced with a Sigma 10 data system (Perkin-Elmer Corp., Norwalk,

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CT, U.S.A.). The data system provided a print-out of the digitally integrated area under the peaks and the retention times for CAPS, CAP and the internal standard. A Perkin-Elmer C-18, ODS-HC-SIL-X-1 reversed-phase column was used. The oven temperature was maintained at 50°C, the flow-rate was 1.5 ml/min, and the detection wavelength was 272 nm.

Reagents

CAP and CAPS were gifts from Parke Davis & Company (Ann Arbor, MI, U.S.A.). 5-Ethyl-*p*-tolylbarbituric acid (ETBA), used as the internal standard, was obtained from Aldrich (Milwaukee, WI, U.S.A.). Sodium acetate, low absorbance grade, was obtained from Matheson, Coleman and Bell (Norwood, OH, U.S.A.). Acetonitrile, ethyl acetate and methanol were HPLC grade and distilled in glass (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). Glacial acetic acid was reagent grade (J.T. Baker, Phillipsburg, NJ, U.S.A.).

The mobile phase was acetonitrile—0.1 *N* sodium acetate (15:85). The pH of this solution was adjusted to 6.4 by the addition of a few drops of glacial acetic acid. 1.0 *N* sodium acetate solution (pH 4.6) was prepared in a similar manner.

Stock standards of CAP, CAPS and ETBA were individually prepared in methanol to yield a final concentration of 1 g/l of each. Working standards were prepared in drug-free serum with appropriate dilutions from the stock solution. The working internal standard (ETBA) was added to the extraction solvent (ethyl acetate) to yield a final concentration of 20 mg/l.

Procedure

Fifty μl control or patient serum, CSF, or urine diluted 1:10 with distilled water, was placed in a 1.5-ml Eppendorf centrifuge tube. An amount of 100 μl 1.0 *N* sodium acetate was added and mixed. 1.0 ml of extraction solvent (ethyl acetate) containing ETBA was then added. The tubes were vortexed vigorously for at least 1 min and then centrifuged in a Brinkman table-top micro centrifuge. The upper organic phase was transferred to a clean glass tube (10 × 13 mm) and evaporated to dryness at 40°C under nitrogen. The samples were sequentially reconstituted with 50 μl methanol, vortexed, and 10 μl were injected onto the column.

RESULTS

Fig. 1A shows a typical chromatogram for blank serum containing the internal standard. The first two peaks appearing in the chromatogram are unidentified artifacts which do not interfere with the determination of CAPS, CAP or ETBA. Fig. 1B illustrates a chromatogram obtained from drug-free serum to which 20 mg/l of CAP and CAPS were added. Fig. 1C is a chromatogram obtained from a patient sample in which the determined concentrations of CAPS and CAP were 12.7 mg/l and 9.4 mg/l, respectively. There were two peaks for CAPS, with retention times of 2.2 (CAPS I) and 2.5 (CAPS II) min. Free CAP and the internal standard had retention times of 3.5 and 5.0 min, respectively. Similar chromatograms were obtained with urine and CSF.

CAP and CAPS were added to drug-free serum to yield concentrations of 5 to 100 mg/l. Concentrations and peak areas were linearly related over this

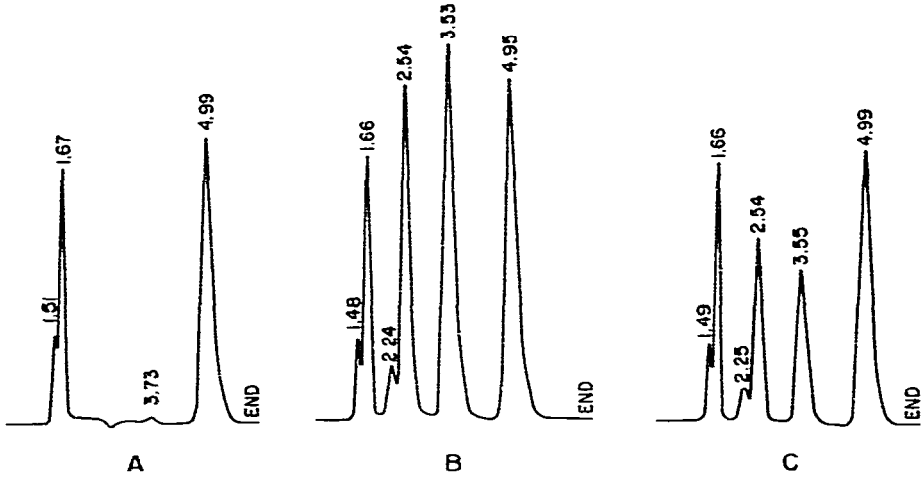

Fig. 1. Typical chromatograms of (A) blank serum containing internal standard; (B) drug-free serum reconstituted with 20 mg/l of CAP and CAPS each; and (C) patient's serum determined as 9.4 mg/l CAP and 12.7 mg/l CAPS. Retention times: CAPS I, 2.2 min; CAPS II, 2.5 min; CAP, 3.5 min; internal standard (ETBA), 5.0 min.

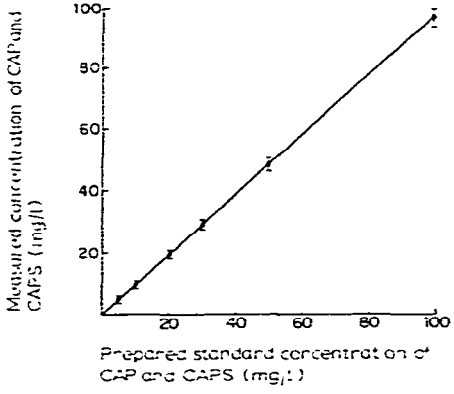

Fig. 2. CAP and CAPS linearity. Mean (●) and range at each concentration for both compounds (n = 5). The standard curves for both compounds are superimposed.

range (Fig. 2). Urine standards of CAP and CAPS prepared in an analogous manner also exhibited linearity over this range.

Within-run precision was evaluated by processing aliquots of a prepared standard serum pool containing 20 mg/l of both CAP and CAPS (Table I). Aliquots of a pool containing CAP and CAPS were individually frozen, and stability studies were conducted by analyzing samples taken from this pool (Table II) for a period of four weeks. The stability studies showed no difference in values obtained from these samples compared to those which were freshly prepared (Table I). Table III indicates the recovery data for different concentrations of both CAP and CAPS. At each concentration, the ratio of the two peak areas of CAPS was constant (20 CAPS I : 80 CAPS II).

TABLE I

PRECISION OF THE ANALYSIS OF FRESHLY PREPARED STANDARDS OF CAP AND CAPS

	Amount added (mg/l)	Mean ± S.D. (mg/l)	*n*
CAP	20	19.9 ± 1.4	22
CAPS	20	20.0 ± 1.2	22

TABLE II

STABILITY OF THE ANALYSIS OF A FROZEN POOL OF CAP AND CAPS OVER A 4-WEEK PERIOD

	Amount added (mg/l)	Mean ± S.D. (mg/l)	*n*
CAP	20	20.0 ± 1.4	14
CAPS	20	19.8 ± 1.9	14

TABLE III

ANALYTICAL RECOVERY OF KNOWN AMOUNTS OF CAP AND CAPS ADDED TO HUMAN PLASMA

Five samples were analyzed at each concentration.

Added (mg/l)		Found (mg/l)		Recovery (%)	
CAP	CAPS	CAP	CAPS	CAP	CAPS
100	100	95	93	95	93
50	50	47	49	96	98
30	30	30	29	100	97
20	20	21	20	105	100
10	10	10.2	10.3	102	103
5	5	5.8	5.4	116	108

DISCUSSION

CAPS standard solutions, prepared in methanol and analyzed according to our procedure, produced a single chromatographic peak. In biological specimens or distilled water, two peaks were always observed (Fig. 1). The sum of the area counts from these two peaks equaled the total area counts obtained from the pure methanolic standards. Therefore, we chose to combine the areas of the two CAPS peaks to calculate the total CAPS concentration in biological fluids.

It has previously been reported [10] that in neutral or acidic solutions CAPS spontaneously forms the cyclic hemi-ortho ester, which exists in equilibrium with the non-cyclic 3-monosuccinate ester of chloramphenicol. We believe that CAPS I represents the cyclic form and CAPS II represents the non-cyclic form of the compound. The ratio of CAPS I to CAPS II (20:80) observed in our

patient samples is consistent with the ratio that has been previously observed in aqueous solutions [10]. As CAPS is eliminated from the serum during a dosing interval, CAPS I always disappears before CAPS II. This suggests that as the major form (3-monosuccinate ester) is hydrolyzed in the body, the equilibrium is shifted and the cyclic form reverts to the 3-ester which is subsequently hydrolyzed. Alternatively, the cyclic form itself may undergo hydrolysis to the active, free chloramphenicol. In either case, the cyclic form exists in lesser amounts and disappears first.

The generally accepted therapeutic range for CAP is 10—20 mg/l. The risk of reversible concentration-related toxicity increases progressively at levels above 30 mg/l. The linearity of this assay up to concentrations of at least 100 mg/l is such that elevated serum CAP concentrations can be accurately determined without the necessity of sample dilution.

CAP and CAPS concentrations in serum determined following refrigeration at 4°C for 24 h were not significantly different from those determined within 6 h of sample collection. However, samples left at room temperature for 24 h or stored refrigerated for longer time intervals showed a variable decrease in the CAPS concentration which did not always correspond to an increase in CAP concentration. The reason for the spontaneous disappearance of CAPS is unknown. CAP and CAPS are stable for at least one month when samples are frozen within 3 h after collection. Because of the apparent instability of CAPS, we recommend that patient samples be analyzed within 24 h from the time of collection or frozen as soon as possible so that erroneous results are avoided.

Since the sample volume used is only 50 μl for both CAP and CAPS, the assay is ideally suited to pediatric patients. This method has been in routine use at this institution for the past year. Our pharmacokinetic studies of CAPS in patients, submitted for publication elsewhere [11], indicate that CAPS is hydrolyzed in a highly variable and unpredictable manner in the body. This hydrolysis is an individual characteristic and may contribute to the lack of attainment of therapeutic levels of chloramphenicol in some patients. Additionally, we have also shown [11] that substantial and variable amounts of an administered dose of CAPS are excreted unhydrolyzed in the urine. We feel that it is important to quantitate CAPS in order to determine if a patient is capable of hydrolyzing the compound during the dosing interval.

In conclusion, a rapid and simple micro-assay for CAP and CAPS has been described. The sensitivity, precision, and accuracy of this method is such that routine clinical laboratories can easily implement it for monitoring levels of CAP and CAPS. Due to the lack of correlation between the administered dose of CAPS and blood levels of free CAP, we recommend that both compounds be measured in all patients receiving CAPS to help in selecting the appropriate dosage schedule and determine if the patient is adequately hydrolyzing the ester.

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