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High-Performance Liquid Chromatographic Assay of Chloramphenicol in Serum

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A new method for the analysis of serum chloramphenicol by reversed-phase, high-performance liquid chromatography (HPLC) is described. The method involves a preliminary extraction of 0.1 ml of serum with ethyl acetate containing an internal standard, chromatography with a reversed-phase C18 microparticulate column with an acetonitrile-acetate buffer mobile phase, and detection by measuring UV absorbance at 270 nm. Assay performance was compared with an existing microbiological assay. The HPLC method demonstrated both increased precision and increased sensitivity. The specificity of the HPLC method was also evaluated. The new method presents an alternative approach to the analysis of clinical specimens.

Chloramphenicol (CAP), the first broad-spectrum antibiotic to be introduced, has enjoyed widespread use over the past three decades. The occasional occurrence of potentially fatal aplastic anemia in patients (20) coupled with the discovery of ampicillin resulted in a dramatic decline in the popularity of this antibiotic. However, recent recognition of the role of anaerobic organisms in the pathogenesis of human disease (7) and the increasing incidence of *Haemophilus influenzae* resistant to ampicillin (2) has led to a resurgence in popularity of this drug. CAP has been recommended as the drug of choice for initial treatment of *H. influenzae* meningitis (27). It is also indicated in the treatment of systemic salmonella infections, infections caused by *Bacteroides fragilis* and rickettsiae, and lymphogranuloma venereum (27).

The devastating aplastic anemia is reported to occur once in every 20,000 to 200,000 patients treated with CAP (28). This reaction appears to be idiosyncratic and not related to dose. Much more common is normocytic suppression of bone marrow, which is usually associated with serum concentrations exceeding 25 μg/ml or prolonged courses of treatment (14, 19, 28). An additional syndrome, consisting of cardiovascular collapse, gastrointestinal distress, respiratory depression, and coma, has been reported in neonates (3, 12, 25), infants (4), and adults (10, 23). This syndrome is most frequently associated with serum CAP concentrations in the range of 40 to 200 μg/ml.

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CAP is eliminated primarily by metabolism in the liver to polar glucuronides (6). Metabolism is impaired in neonates (25) and in adults with liver disease (1). Therefore, in these populations, monitoring of serum concentrations is essential for the determination of optimal dosing. We have developed a reversed-phase high-performance liquid chromatographic (HPLC) method which is specific for CAP. A comparison of this method with an existing microbiological assay (21) and an evaluation of the specificity of the newly developed method are the subjects of this report.

MATERIALS AND METHODS

Analytical procedure. One hundred microliters of serum was added to 0.1 ml of 1.0 N sodium acetate buffer, pH 4.6. To this was added 1.0 ml of ethyl acetate containing 12.5 μg of 5-ethyl 5-*p*-tolylbarbituric acid (99+%; Aldrich Chemical Co., Milwaukee, Wis.) per ml as an internal standard. The mixture was shaken for 3 min (horizontal Eberbach shaker, high speed) in 1.5-ml polypropylene micro test tubes. The samples were centrifuged for 30 s (8,000 × *g*) in a Brinkmann 3200 centrifuge. Approximately 0.8 ml of the organic phase was transferred to a glass test tube (12 by 75 mm). The organic phase was evaporated to dryness under nitrogen at 40°C (5 min). The residue was redissolved with 0.2 ml of methanol; 0.02 ml of this solution was injected (Rheodyne 0.020-ml loop injector) into the HPLC (Laboratory Data Control Div., Milton Roy Co., Riviera Beach, Fla.; Constametric I pump and Spectromonitor I). The chromatographic column used was a 25-cm *u* Bondapac C18 (PN-27324; Waters Associates, Inc., Milford Mass.). Column temperature was maintained at 40°C. The mobile phase was 25% acetonitrile (Photrex, J. T.

Baker Chemical Co., Phillipsburg, N.J.) in pH 6.0, 0.1 N acetate buffer. Solvent flow was 2.0 ml/min. The detector (variable wavelength) measured absorbance at 270 nm (0.04 absorbance unit, full scale) with a time constant of 0.5 s. Typical chromatograms are shown in Fig. 1 for injections of plasma standards of 10, 20, and 40 μg of CAP per ml.

Standards were prepared with pooled serum to contain 10, 20, 30, and 40 μg of CAP (Chloromycetin, analytical standard; Parke, Davis & Co., Detroit, Mich.) per ml. These standards were frozen in 0.5-ml portions and were stable for several months. A four-point standard curve was prepared daily by plotting the ratio of the CAP peak height to that of the internal standard for each standard concentration. Linear regression analysis of calibration curve data indicated no significant deviation from linearity ($R = 0.998$ to 0.999 on 5 separate days). In addition, intercept values did not differ significantly from zero ($P > 0.05$ on 4 days, $P > 0.02$ on 1 day). The ratios observed for unknown samples were inserted into the regression equation, which was solved for the test concentration.

Assessment of specificity. Aqueous solutions were prepared containing 100 μg of the following drugs per ml: gentamicin, tobramycin, amikacin, carbenicillin, ticarcillin, ampicillin, oxacillin, dicloxacillin, penicillin G, cephalothin, erythromycin, and clindamycin. Solutions were also prepared containing 10 μg of the following per ml: CAP-succinate, CAP-palmitate, internal standard, thiamphenicol, theophylline, and caffeine. These solutions were injected directly into the HPLC. Compounds producing peaks are listed in Table 1, which shows retention time (minutes) and peak height (millimeters). These solutions were then extracted as described above and reinjected to determine potential interference with the assay. Direct injection of CAP (four times) was compared to postextraction results (four times) to assess the extraction recovery.

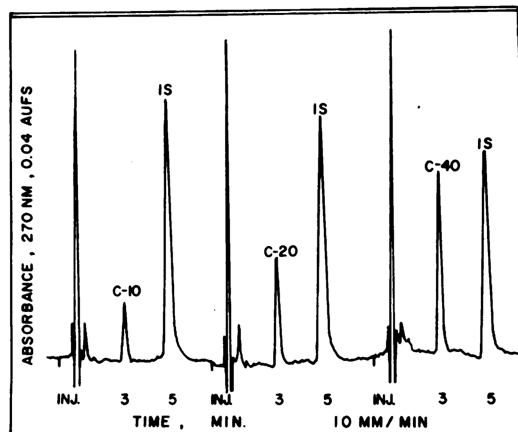

FIG. 1. *Typical chromatographic output resulting from analysis of standards prepared in blank serum containing 10, 20, and 40 μg of chloramphenicol per ml (C-10, -20, and -40). Abbreviations: IS, internal standard (5-ethyl 5-p-tolylbarbituric acid); INJ, injection; AUFS, absorbance units, full scale.*

The specificity of the assay for CAP in the presence of metabolites was also investigated. Urine containing CAP-glucuronide was diluted 1:20 with 0.4 M acetate buffer, pH 4.5. The specimen was divided into two aliquots of 4.0 ml each. To one aliquot 5,000 U of β-glucuronidase (Ketodase; Warner-Chilcott Laboratories, Morris Plains, N.J.) was added. Both samples were incubated in a water bath overnight at 38°C. Both the treated sample and the control sample were injected directly into the chromatograph.

Acid and alkaline breakdown products which are also known to exist as metabolites in humans were studied (22). Aqueous samples of CAP were incubated at neutral pH, pH 1 (HCl), and pH 12 (NaOH) at 60°C for 5 h. Hydrolysis of the dichloroacetamido group is the only significant reaction which occurs below pH 7. This reaction produces a basic product with a pK_a of 8.6 (22). Alkaline conditions result in hydrolysis of covalently bound chlorine molecules of the dichloroacetamido moiety with subsequent production of a more water-soluble alcohol. Secondary decomposition may also occur at alkaline pH (22). Samples taken from all three solutions were injected directly into the chromatograph.

Reduction of the *p*-nitro to an amino group produces a class of metabolites referred to as arylamines. The amino group is a weak base, pK_a 5.1 (information supplied by Park Davis & Co., Detroit, Mich.). Authentic arylamine metabolites were not available for study, nor could they be easily synthesized. These compounds are only present in significant concentrations in the sera of patients with severe renal impairment (9). A means of preventing interference by these compounds will be suggested in Discussion.

Comparison of HPLC with microbiological assay. Ten pooled-serum samples were prepared in the following concentrations: 2.5, 5, 7.5, 10, 15, 20, 25, 30, 35, and 40 μg of CAP per ml; aliquots of these specimens were stored at −20°C until analysis. Assay was performed with Brewer thioglycolate medium plus 1.5% agar (Difco Laboratories, Detroit, Mich.) and *Bacillus subtilis* spores (ATCC 6633) according to the method of Smith et al (21). All samples were assayed on 5 consecutive days. Zones of inhibition were measured in triplicate, and mean values were determined. The mean diameters for the 10-, 20-, 30-, and 40-μg/ml samples were used to generate a curve by linear least squares fit of zone diameter versus the logarithm of the standard concentration. The equation of the regression curve was used to estimate all 10 spiked concentration values.

Similarly, the 10 spiked samples were assayed on 5 separate days by HPLC, using the 10-, 20-, 30-, and 40-μg/ml spikes to generate a least squares standard curve. This curve was then used to estimate the concentrations of all 10 spikes based on observed peak height ratios. In addition, the 7.5- and 25.5-μg/ml spiked samples were assayed five times in the same run to evaluate the within-run precision of the assay.

RESULTS

Specificity. All compounds which produced peaks after injection into the HPLC were extracted as authentic samples and reinjected. The

results of this study are shown in Table 1. Although several compounds produced peaks, either the peaks occurred at times which did not coincide with that of either CAP or the internal standard or the compounds were not extracted by ethyl acetate. For example, gentamicin, which produced a small peak at the same time as CAP, was not extracted and, thus, would not interfere with the assay. The recovery of CAP after extraction, evaporation, and reconstitution with methanol was found to be 96.4 ± 3.6%.

Injection of dilute untreated urine produced a large peak near the solvent front (polar), with a much smaller peak at the location of CAP. Incubation with β-glucuronidase resulted in removal of the early, polar peak and a marked increase in the CAP peak. Thus, the glucuronide metabolite, if extracted, will produce an early peak which will not interfere with analysis. Both alkaline and acid hydrolysis produced marked reductions in the CAP peak when compared with the sample incubated at neutral pH. In both cases, a peak appeared near the solvent front, as would be expected due to the polar nature of the products formed. No other peaks appeared on the chromatograms. It would, therefore, appear that the dehalogenated and the hydrolyzed products will not interfere with the assay.

Comparison of HPLC and microbiological assay. The results of comparison of the HPLC and microbiological assays in the analysis of the 10 spiked samples on 5 separate days are shown in Fig. 2. Overall correlation between the two methods ($N = 50$) was excellent ($R = 0.966$, $P << 0.001$). Regression analysis of the microbiological assay data compared with spike values revealed a regression line with a slope of 1.08 ± 0.038 (standard error [SE]), which was significantly different from 1 ($P < 0.05$), and an intercept equal to −2.74 ± 0.85 (SE), which was significantly different from 0 ($P < 0.01$). The correlation coefficient was 0.972 ($P << 0.001$). The 95% confidence intervals for assay results for 10-, 20-, and 30-μg/ml samples were 1.3 to 14.8, 12.0 to 25.5, and 22.8 to 36.3 μg/ml, respectively. Analysis of spike versus HPLC results revealed a regression line with a slope of 1.05 ± 0.02 (SE), which was significantly different from 1.0 ($P < 0.01$), and an intercept of −1.83 ± 0.34 (SE), which was significantly different from 0 ($P < 0.001$). The correlation coefficient was 0.995 ($P << 0.001$). The 95% confidence intervals for assay results for 10-, 20-, and 30-μg/ml samples were 6.0 to 11.4, 16.5 to 21.9, and 26.9 to 32.4 μg/ml, respectively. Regression lines and the means and standard deviations (SD) of the five

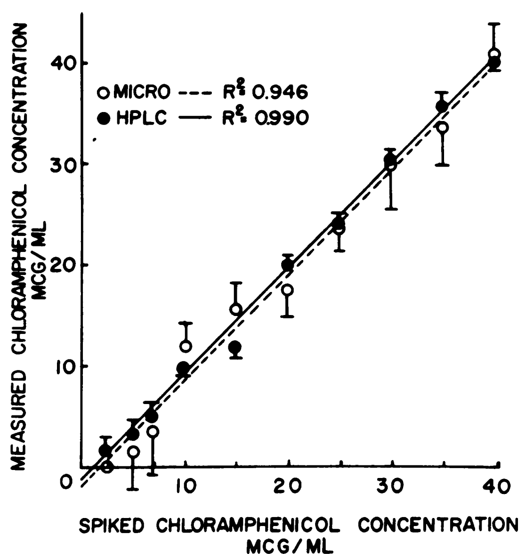

FIG. 2. *Mean and SD of quintuplicate results for HPLC (●) and microbiological (O) analysis of 10 spiked serum samples. Regression lines and coefficients of determination (R^2) are shown for HPLC (—) and microbiological assay (----).*

TABLE 1. *HPLC specificity study*

Compound injected	Concn (μg/ml)	Direct: Retention time (min)	Direct: Peak height (mm)	Postextraction[a]: Retention time (min)	Postextraction[a]: Peak height (mm)
Caffeine	10	2.0	163	2.0	80
Cephalothin	100	2.4	270	2.4	29
CAP	10	3.2	62	3.2	30
CAP-succinate	10	2.2	40	2.2	20
Dicloxacillin	100	5.6	16	—[b]	—
Gentamicin	100	3.2	10	—	—
Internal standard	10	4.8	4	4.8	2
Oxacillin	100	3.0	15	—	—
Theophylline	10	1.4	127	1.4	40

[a] Reconstitution postevaporation results in dilution of samples by 50%.
[b] —, Not detected.

replicate assays for all spiked samples are shown in Fig. 2.

Within-day replicate HPLC analysis of the 7.5- and 25.0-μg/ml spikes ($N = 5$) produced the following: 6.7 ± 0.4 (±1 SD, coefficient of variation [CV] = 5.9%) and 25.8 ± 1.0 (±1 SD, CV = 3.9%). These results demonstrated increased precision when compared to results obtained on separate days: 6.3 ± 0.8 (±1 SD, CV = 12.7%) and 24.4 ± 1.5 (±1 SD, CV = 6.1%).

DISCUSSION

The choice of analytical method for use in the performance of analysis of drug concentrations in serum will depend to a large extent on the background and expertise of the investigator. Instrument availability, sample volume, and required minimum turnaround time will also be considerations. This study presents an alternative approach to the analysis of serum CAP concentrations in patient samples. The proposed method offers the advantage of being easily adapted to existing HPLC instrumentation. By slight modifications of mobile phase and detection wavelength, this method could be employed on instruments being used for the analysis of theophylline (17) and antiepileptic drugs (8). A fixed-wavelength detector at 254 nm could be used, since detector response to CAP declines by only 44% as wavelength is changed from 270 to 254 nm. This change in wavelength produced an 83% increase in response to the internal standard. The common use of these drugs and requirement for their analysis in pediatric institutions makes multiple drug analyses on one HPLC attractive.

Several methods have been previously described for the analysis of CAP in serum and other biological fluids. The microbiological assay is relatively simple to perform and is commonly used (21). Generally, overnight incubation of plates is required, which results in less-than-optimal assay turnaround. Cross-reaction with concurrently administered antibiotics presents a serious problem, as does the poor sensitivity and precision of the microbiological assay (21). A recent modification, using *Clostridium perfringens*, has reduced the incubation time to 2 to 3 h (13). This method, which measures inhibition of hemolysis, appears to be sensitive to CAP concentrations above 2 μg/ml and is not affected by the presence of high concentrations of gentamicin in the sample. However, colony maintenance and plate preparation are complex, and cross-reactivity with penicillins, cephalosporins, clindamycin, and tetracyclines remains a problem. A spectrophotometric method has also been described (16) in which a yellow color develops upon reaction of CAP with isonicotinic acid hydrazide after extraction from serum into isoamyl acetate. This method appears to be specific for CAP in the presence of many other antibiotics, CAP esters, and glucuronide metabolite. The procedure is time consuming (2 to 4 h), requires a large sample volume (1.0 ml of serum), and has a sensitivity limit of approximately 5 μg/ml. Gas-liquid chromatographic methods have been reported (15, 18). These methods generally require derivatization of CAP before analysis. The destructive nature of the method of detection prevents further study of separated compounds. Finally, enzymatic assays for CAP have recently been presented (5, 11). Aside from the technical problems of producing the acetyltransferase enzyme and the requirement for a liquid scintillation counter, these assays appear to offer excellent precision, accuracy, sensitivity, and specificity.

HPLC offers an alternative approach to drug analysis. Formation of derivatives of compounds is rarely required. The diversity of methods of separation of solutes has resulted in widespread application of HPLC to the analysis of drugs in biological specimens. The lipid solubility and UV absorbance of CAP make it a prime candidate for reversed-phase HPLC analysis. One method has been described in which HPLC was used to quantitate intermediates in CAP synthesis (24); more recently, reversed-phase HPLC has been applied (26). Initial evaluation of this method revealed a significant interference between CAP and theophylline.

The HPLC method presented here was clearly superior to the microbiological method in terms of precision, accuracy, and sensitivity. Comparison of specificity was not directly performed, since it is well recognized that the microbiological assay is not specific for CAP. It would appear that no commonly used antibiotics will interfere with the HPLC assay. The studies of metabolites described above indicate that the HPLC assay is specific for CAP.

Only the arylamine metabolites were not studied directly. These products may have chromatographic retention characteristics which differ from the parent compound, but this cannot be proven without authentic material. These metabolites can most likely be removed as a potential interference by a slight modification of the extraction procedure. The use of 1.0 M disodium citrate buffer (pH 1.5), which, when mixed with an equal volume of serum, produces a pH of 2.5 to 3.0, will prevent the extraction of significant amounts of arylamine (weak base, pK_a 5.1). This buffer does not alter the recovery of CAP or the internal standard, nor does it

cause significant degradation of CAP over 30 min at room temperature. It may alter the recoveries of the penicillins (weak acids, $pK_a \cong 3.0$) studied above. This buffer is not used routinely, as it produces increased recoveries of endogenous weak acids present in serum. Although these substances do not interfere with analysis, it is feared that they may have an adverse effect on column life.

The CV of greater than 10% for between-day analysis of the 7.5-μg/ml spiked sample indicates that this value is the lower limit for precise quantitation of specimens by the described method. Reducing the volume of methanol used to reconstitute the residue of the organic phase could be expected to lower this limit. Although this level of sensitivity is above that observed in enzymatic (5, 11) and gas chromatographic (14, 18) assays, it is well below concentrations of clinical interest. The precision of the presented HPLC assay is also within acceptable limits for clinical application. In conclusion, the presented method appears to be specific for CAP and is of sufficient sensitivity, precision, and accuracy for clinical application.

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