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Note

Short-column liquid chromatographic assay for caffeine and chloramphenicol in serum

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Caffeine and chloramphenicol are two drugs commonly used in the neonatal population. Caffeine is used as a respiratory stimulant for the treatment of apnea of prematurity. Chloramphenicol is a broad-spectrum antibiotic that is particularly effective in the treatment of ampicillin-resistant *Hemophilus influenzae* meningitis [1]. While the therapeutic utility of these agents is well documented, the adverse effects which may occur, particularly at elevated blood concentrations, has prompted the need for close monitoring. In our institution a subpopulation of premature infants are treated with intravenous chloramphenicol and caffeine during high-frequency ventilation. This specific application has prompted us to develop a combined assay for both compounds.

Many chromatographic procedures have appeared in the literature for the determination of either caffeine and its metabolites [2,3] or chloramphenicol in serum. However, to our knowledge, no reports have focused on the simultaneous measurement of these drugs in serum. The determination of caffeine and its pharmacologically active metabolites may be limited to the measurement of parent compound (caffeine) only, since N-demethylation is diminished in the neonate [4]. In one procedure for the measurement of theophylline and caffeine [5], retention times for chloramphenicol are reported as 28.07 and 35.08 min at flow-rates of 5 and 4 ml/min, respectively. While the caffeine retention time is approximately 5–6 min, the long development times for chloramphenicol would not lend the procedure to routine operation. In several pro-

cedures for determination of chloramphenicol in serum [6–9]. caffeine is only mentioned as not interfering with the measurement of the drug of interest.

We report here the simultaneous measurement of caffeine and chloramphenicol in serum using a 3.3-cm-long C_{18} column packed with 3-μm particles. Through the use of the short column, the separation of the two drugs is achieved in a relatively short time as compared to longer, standard-sized columns. The advantage to this procedure is the elimination of two different procedures, especially when test volume is low. In addition, since both drugs can be measured from one sample, less blood is required from neonates who may have been treated with both of these agents.

EXPERIMENTAL

Apparatus

Chromatography was performed using a Perkin-Elmer Series 2 liquid chromatography pump (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a Rheodyne 7105 injector (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl sample loop. The compounds were detected by a Perkin-Elmer LC-75 variable-wavelength detector at 278 nm and recorded on a Fisher Recordall Series 5000 chart strip recorder (Fischer Scientific, Pittsburgh, PA, U.S.A.) with a chart speed of 0.5 cm/min.

The high-performance liquid chromatography (HPLC) column was a Perkin-Elmer 3.3 cm $\times$ 4.6 mm reversed-phase C_{18} column packed with 3-μm particles. A Rheodyne column inlet filter with a 0.5-μm filter element was used to protect the column from particulates.

Reagents

Sodium acetate, sodium hydroxide and acetic acid were purchased from Mallinckrodt (St. Louis, MO, U.S.A.), chloramphenicol and caffeine from Sigma (St. Louis, MO, U.S.A.), 4-nitroacetanilide from Eastman Kodak (Rochester, NY, U.S.A.) and isopropanol from Fisher Scientific.

Preparation of mobile phase

The mobile phase consisted of 2% isopropanol in 0.1 M sodium acetate buffer, pH 5.0. The pH was adjusted by the addition of 1 M sodium hydroxide or 1 M acetic acid. Prior to mixing with isopropanol, the acetate buffer was filtered through a 0.45-mm filter (Gelman Sciences, Ann Arbor, MI, U.S.A.). After mixing of buffer and isopropanol, the mobile phase was degassed using vacuum and ultrasonication.

Standards preparation

A standard solution of chloramphenicol in water was made to a concentration of 25 mg/ml. A 10-ml aliquot of this solution was then added to a Sigma

CAF-200 standardized caffeine vial containing 192 mg of caffeine. The final concentration of the caffeine standard was 19.2 mg/ml.

A 1% stock solution of 4-nitroacetanilide was prepared in acetonitrile. A working solution of internal standard was prepared by diluting 2.2 ml of the stock standard solution to 100 ml with spectral-grade ethyl acetate. All standards were kept refrigerated in glass containers and were stable for at least three months. The serum controls used for the evaluation of interfering substances were Gilford Level I and Level II controls (Gilford, Irvine, CA, U.S.A.).

Procedure

A 100-μl aliquot of standard, sample or control was placed into a polypropylene microcentrifuge tube. A 500-μl aliquot of 4-nitroacetonilide working internal standard in ethyl acetate was added to the tube. The tube was capped, vortex-mixed for 30 s and then centrifuged at 7000 g for 1 min. With a disposable glass pipette, the top organic layer was removed and placed in a 75 mm $\times$ 12 mm glass tube. The organic layer was evaporated with a stream of nitrogen. The dried residue was reconstituted with 100 μl of mobile phase, and 10 μl were injected into the chromatograph.

RESULTS AND DISCUSSION

A two-point linear calibration curve was prepared for each drug (caffeine and chloramphenicol) by plotting the ratios of standard peak heights to internal standard (I.S.) peak heights versus standard concentration. The following equation summarizes the above relationship:

$$\text{Concentration of sample (mg/ml)} = \frac{\text{sample/I.S. peak-height ratio}}{\text{slope of calibration curve}}$$

Fig 1A, B and C are representative chromatograms obtained for standards, a patient receiving caffeine and a patient receiving chloramphenicol, respectively. Analysis of a blank sample did not show any interfering peaks. While the 10-min retention time is rather long for a high-speed column, the difference in chromatographic behavior of the two drugs dictates the extended time. Using the same mobile phase, a retention time of 21 min is obtained when the three components are separated on a 15-cm-long column with 10-μm particles. Similarly, a 25-cm-long column with 5-μm particles produced a retention time of 77 min. A shorter time could be obtained using gradient elution, but equilibration of the column between runs would add to the total time. A mobile phase with a higher concentration of isopropanol could also shorten retention time; however, merging of the caffeine peak with the solvent front might pose a problem. Accordingly, use of the high-speed column with a conventional retention time was accepted.

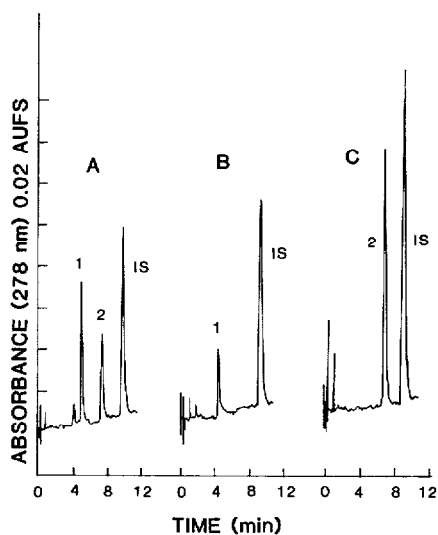For the reversed-phase separation of caffeine, previous procedures have uti-

Fig. 1. Separation of caffeine, chloramphenicol and internal standard (4-nitroacetanilide) from the extraction of (A) a standard solution containing 19.2 μg/ml caffeine and 25.0 μg/ml chloramphenicol, (B) a patient serum sample with a caffeine concentration of 8.8 μg/ml and (C) a patient serum sample with a chloramphenicol concentration of 42.8 μg/ml. Extraction and chromatographic conditions are described in the text. Peaks: 1 = caffeine; 2 = chloramphenicol; IS = internal standard.

lized a mobile phase consisting of approximately 10% organic modifier [5,10]. The reversed-phase separation of chloramphenicol generally uses mobile phases containing approximately 25–35% organic content [6–8, 11–14]. In one procedure [15] a 60% organic content was used; however, octanesulfonic acid was used as an additive in this method. The mobile phase in our method uses 2% isopropanol as the organic solvent. Use of the more lipophilic solvent requires less of the modifier. As a result, a reduction in solvent expense is realized. In addition, use of the short column does not cause pressure problems such as those encountered when using isopropanol with conventional columns. A flow-rate of 2 ml/min can easily be increased to 3 ml/min without significant degradation of chromatographic performance.

The internal standard used was 4-nitroacetanilide. This internal standard has been successfully used in previous reports [6,11] for the analysis of chloramphenicol. Mephenisin [13,15], benzoic acid [12], sulfamethoxazone [14], 5-ethyl-5-p-tolylbarbituric acid [8] and 2,4-dinitroacetanilide [7] have also been used as internal standards in previous methods for the measurement of chloramphenicol. While mephenisin and 5-ethyl-5-p-tolylbarbituric acid may have adequate absorptivity at 278 nm [7], none of these were investigated since 4-nitroacetanilide performed satisfactorily. β-Hydroxyethyltheophyl-

line, which is generally used as the internal standard in caffeine procedures [5,10,16], was not used since it eluted near the solvent front.

For the extraction of caffeine and chloramphenicol, several techniques were investigated. Firstly, protein precipitation and internal standard addition via acetonitrile were investigated as previously performed [6,11,12]. Initially, this method worked very well; however, column degradation was found to occur prematurely. This was most likely due to incomplete protein removal [7] and subsequent irreversible adsorption of these proteins onto the column. The salt-induced phase separation technique of Ryan et al. [7] was attempted; however, low recoveries of caffeine were obtained. Extraction with dichloromethane was investigated [10,14], but again recoveries for caffeine were low. Finally, in an approach similar to that of Soldin et al. [8], ethyl acetate was successfully utilized as the extraction solvent for both caffeine and chloramphenicol.

Once extraction and chromatographic conditions were established, performance characteristics of the method were determined. For linearity, an upper limit of 100 μg/ml was established for both drugs. The linear equation for caffeine concentration versus the caffeine/I.S. peak-height ratio was $y = 0.10x + 0.06$ with a correlation coefficient of 0.9953. Similarly, for chloramphenicol, the linear equation was described as $y = 0.06x + 0.05$ with a correlation coefficient of 0.9947. Concentrations of both drugs were tested from 10 to 100 μg/ml in increments of 10 μg/ml. No concentrations greater than 100 μg/ml were analyzed. While it may be rare that a concentration greater than 100 μg/ml would be obtained for either drug, simple dilution of the serum with water prior to analysis would facilitate a concentration in the established linear range.

Within-run and between-run reproducibility studies were obtained using the spiked value of the control preparations. The within-run reproducibility for caffeine produced a mean $\pm$ S.D. of 17.7 $\pm$ 1.0 μg/ml and a coefficient of variation of 5.6% ($n = 10$). For chloramphenicol the mean $\pm$ S.D. was 15.8 $\pm$ 0.8 μg/ml and the coefficient of variation was 5.1% ($n = 10$). The between-run precision for caffeine produced a mean $\pm$ S.D. of 18.8 $\pm$ 0.8 μg/ml and a coefficient of variation of 4.3% ($n = 9$) and for chloramphenicol a mean $\pm$ S.D. of 17.2 $\pm$ 1.3 μg/ml and a coefficient of variation of 7.6% ($n = 9$).

Recoveries for caffeine were determined to be 99.2 $\pm$ 5.9% at a level of 7.7 μg/ml ($n = 5$). For chloramphenicol, recoveries of 89.2 $\pm$ 11.4% were obtained at a level of 10.0 μg/ml ($n = 5$).

Table I lists those drugs that did not interfere with the measurement of caffeine, chloramphenicol or the internal standard. Interference by these drugs was tested by extracting a commercial control containing therapeutic levels of the drugs. In addition, several xanthine derivatives were examined for potential interference. Table II lists the retention times for the compounds of interest as well as those of the interference candidates. All of the xanthines examined eluted prior to the caffeine peak. The pro-drug of chloramphenicol,

TABLE I

DRUGS THAT DO NOT INTERFERE WITH CAFFEINE AND/OR CHLORAMPHENICOL

Acetaminophen	Methotrexate
Amikacin	N-Acetylprocainamide
Amitriptyline	Netilmicin
Carbamazepine	Nortriptyline
Cyclosporine	Phenobarbital
Digoxin	Phenytoin
Desipramine	Primidone
Disopyramide	Procainamide
Ethosuximide	Quinidine
Gentamicin	Salicylate
Imipramine	Theophylline
Lidocaine	Tobramycin
Lithium	Valproate
	Vancomycin

TABLE II

RETENTION TIMES FOR CAFFEINE- AND CHLORAMPHENICOL-RELATED COMPOUNDS

Compound	Retention time (min)
Caffeine	3.85
Chloramphenicol	6.32
4-Nitroacetanilide	8.27
Uric acid	0.31
1-Methyluric acid	0.50
1,3-Dimethyluric acid	0.85
Theobromine	1.04
1,7-Dimethylxanthine	1.46
Theophylline	1.75
Dyphylline	1.78
Chloramphenicol 3-monosuccinate	9.98

chloramphenicol 3-monosuccinate, was also evaluated for interference. This compound eluted after the internal standard and would cause a problem only if the next sample was prematurely injected. The 1-succinate ester of chloramphenicol was not evaluated. This compound may interfere with the internal standard since it has been shown in another study [8] to elute between chloramphenicol and the 3-succinate ester.

CONCLUSIONS

The described method provides a quick, simple and reliable method for the simultaneous measurement of caffeine and chloramphenicol. Through the use

of the short column in a non-high-speed mode, separation of the two drugs can be achieved isocratically in approximately 10 min. Use of 4-nitroacetanilide as the internal standard has proven to be satisfactory, although other choices may perform equally well. The limited efficiency of the short column coupled to the relatively high speed of operation may provide for the separation of many pairs or even groups of dissimilar drugs that have in the past required long retention times or gradient elution.

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