Journal of Chromatography, 417 (1987) 111–119
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3613

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CHLORAMPHENICOL AND FOUR ANALOGUES USING REDUCTIVE AND OXIDATIVE ELECTROCHEMICAL AND ULTRAVIOLET DETECTION

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(First received October 28th, 1986; revised manuscript received January 20th, 1987)

SUMMARY

A high-performance liquid chromatographic procedure is described for the separation, quantitation and identification of chloramphenicol, dehydrochloramphenicol, nitrophenylaminopropanedione, nitrosochloramphenicol and aminochloramphenicol. An isocratic reversed-phase system with ultraviolet and electrochemical detectors in tandem was assembled and used. The system was constructed with special accommodation to enable us to use the electrochemical detector in both reductive and oxidative modes. Retention characteristics, hydrodynamic voltammograms under reductive and oxidative conditions and ultraviolet absorbance are reported. Applicability of the procedure to biological fluids was demonstrated by separation and detection of chloramphenicol after incubation with human blood.

INTRODUCTION

Chloramphenicol (CAP), a broad-spectrum antibiotic, is commonly associated with reversible bone marrow suppression and rarely with a devastating irreversible bone marrow aplasia or aplastic anemia. Understanding the mechanism

0378-4347/87/$03.50

of toxicity caused by CAP is a major subject that has been of interest to us for many years [1–5]. Based on our extensive studies we have postulated that the *p*-nitro group of CAP undergoes reduction yielding toxic and highly reactive intermediates which result in cell damage at different subcellular levels [6–8]. Recently, we have observed that some metabolites of CAP, known to be produced by common intestinal bacteria, exhibit much greater cytotoxicity in vitro than the parent molecule. The toxicity of these compounds may be related to further metabolic transformation by the target cell. Accordingly, a detailed study of the metabolism of CAP and its derivatives by mammalian cells, particularly bone marrow cells, becomes important for elucidating pathogenetic mechanism in CAP-induced bone marrow injury. Critical to the success of such studies is the availability of methods directed at the detection of metabolic products.

CAP has been previously quantitated in biological fluids using conventional high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [9–13]. Such methods are limited by the UV absorption and extinction coefficient of the compounds studied. Other less specific techniques have also been described (reviewed in ref. 14). Because of this limitation, a more sensitive and specific mode was sought for our metabolic studies. In this paper we describe for the first time the use of an HPLC system equipped with special dual detection system: UV and electrochemical detection (ED) designed to be utilized in both reductive and oxidative modes. The availability of these two ED modes in tandem with the conventional UV detector increases exponentially the selectivity and sensitivity of the system. This did not only enhance the sensitivity and selectivity but also provided a powerful basis for the identification of unknown metabolites. Data on the separation, UV absorption, hydrodynamic voltammograms and simultaneous multiple detection for CAP, its nitro, nitroso and amino analogues are reported.

EXPERIMENTAL

Instrumentation

An isocratic HPLC system was assembled in our laboratory from commercially available components which were modified to be compatible for use with reductive ED. It consisted of a Constametric III pump and a SpectroMonitor 3000 variable-wavelength UV detector (LDC/Milton Roy, Riviera Beach, FL, U.S.A); an LC-4B electrochemcial detector equipped with a glassy carbon working electrode, a BAS-LC 100 consisting of a stand, column bracket, pressure gauge, sample injector, pulse dampner, and a BAS Biophase ODS 5-μm column (Bioanalytical Systems, West Lafayette, IN, U.S.A.); and a Model 7125 syringe loading sample injector equipped with 200 μl-loop (Rheodyne, Cotati, CA, U.S.A.).

The column effluent was connected to the UV detector, then to the electrochemical detector in tandem using 0.25 mm I.D. stainless-steel tubing and low-dead-volume Valco unions.

The mobile phase was degassed and kept warm in a 2-l round-bottom flask using a heating mantle and a rheostat to maintain the temperature at approximately 45 °C. Both the mobile phase and the samples were purged with helium,

grade 99.999%, which was filtered through a CRS hydrocarbon trap (Chemical Research Supplies, Addeson, IL, U.S.A.). Two Nupro needle valves (Nupro, Willoughby, OH, U.S.A.) were used to control the helium flow to the mobile phase and the Rheodyne injector. A Teflon stopper was machined to fit the 24/40 round-bottom flask. Four holes were dilled to fit the following tubing very snugly: (1) 3.2-mm stainless steel tubing to which a fitted 45-μm stainlesss steel filter was attached; this tube is for solvent intake to the pump; (2) a 1.6-mm Teflon tubing for solvent return; this tubing is connected to the exit part of the electrochemical detector for recycling the mobile phase; (3) a 1.6-mm stainless-steel tubing connected to the helium for bubbling helium through the mobile phase; (4) a 1.6-mm stainless steel tubing immersed in a 15-ml test tube filled with mobile phase and stoppered to accommodate outgoing helium; the purpose of this test tube is to maintain helium pressure on mobile phase.

Sample (0.7 ml) degassing was accomplished as in ref. 15 with some modification. Helium was directly connected to vent No. 5 of the Rheodyne sample injector. A 1-ml capacity Kloehn special gas-tight syringe (Kloehn, Brea, CA, U.S.A.) was modified by drilling a 1.6-mm hole at the top of the barrel and having that part of the barrel supported by a stainless-steel ring (Chromtec, Miami, FL, U.S.A.). The helium was bubbled through the sample with the injector in inject position. A long needle was used to allow tilting the syringe about 90° and keeping it in the upright position during degassing. The 200-μl loop was overloaded by injecting a 0.6-ml sample.

All tubing used in the system were of 316 stainless steel including the connections within the Constametric III pump. The only Teflon tubing remaining in the system was the outgoing effluent from the electrochemical detector which was either discarded during chromatography or returned to the mobile phase for recycling when no injections were being made onto the column.

The output of both UV and electrochemical detectors were connected to a Linear, double-pen recorder (Linear Instruments, Reno, NV, U.S.A.) for almost simultaneous recording of peaks (lag time between the two pens was determined to be 15 s). The electrochemical detector output was also connected to a Model HP 3392A computing integrator (Hewlett-Packard, Avondale, PA, U.S.A.) for quantitative analysis. The data in the figures are representative of the separation, detection and hydrodynamic voltammograms as recorded in three reproducible experiments.

Chromatographic conditions

The HPLC conditions for separation and detection of CAP and its analogues were as follows: column, 5-μm ODS (25 cm$\times$4.6 mm); mobile phase, 20 m*M* monochloroacetic acid, 1 m*M* Na_2EDTA, 14.6 m*M* sodium acetate in water–*n*-propanol (92:8) [16]; sample size, 200 μl; flow-rate, 1.5 ml/min; detection, dual UV and electrochemical detection; a glassy carbon electrode versus Ag/AgCl reference electrode was used in both oxidative and reductive ED modes (see figures for details); sensitivity, UV=0.02 a.u.f.s. and ED=100 nA f.s; temperature, ambient; chart-speed, 30 cm/h.

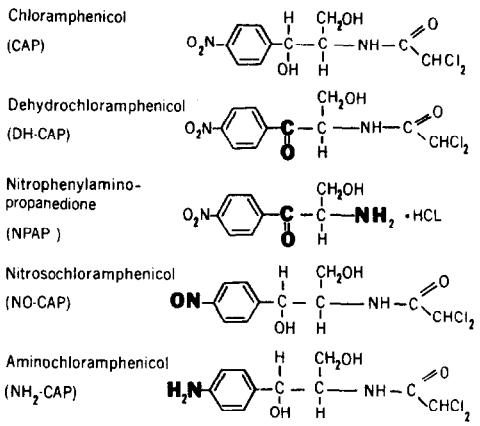


Fig. 1. Structures of chloramphenicol (CAP) (1-*p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol), dehydrochloramphenicol (DH-CAP) (4′-nitro-2-dichloroacetamido-3-hydroxypropiophenone), nitrophenylaminopropanedione (NPAP) [(*R*)-4′-nitro-2-amino-3-hydroxypropiophenone, hydrochloride], nitrosochloramphenicol (NO-CAP) (1-*p*-nitrosophenyl-2-dichloroacetamido-1,3-propanediol) and aminochloramphenicol (NH_2-CAP) (1-*p*-aminophenyl-2-dichloroacetamido-1,3-propanediol).

Preparation of drugs and blood

CAP was purchased from Parke-Davis, Division Warner-Lambert (Morris Plains, NJ, U.S.A.) Nitrophenylaminopropanedione (NPAP), nitrosochloramphenicol (NO-CAP), dehydrochloramphenicol (DH-CAP) and aminochloramphenicol (NH_2-CAP) were synthesized by Zambon Labs. (Milan, Italy). Other reagents were of HPLC grade. All drugs were freshly prepared before every experiment. Stock solutions were dissolved in dimethylsulfoxide (DMSO), then diluted in the mobile phase prior to injection. Injected samples were filtered through 25-mm nylon filter units with pore size of 0.45 μm purchased from Lida Manufacturing (Bensenville, IL, U.S.A.).

Freshly collected blood from donors was used as biological fluid. Aliquots of 1.5 ml were incubated in a Dubnoff metabolic shaker with 100 μM chloramphenicol at 37 °C. After 60 min, 1.5 ml ice-temperature perchloric acid (0.35 *M* final concentration) were added. The acid would stop any possible metabolic reaction that might have occurred in time. The resulting mixture was then centrifuged for 4 min in an Eppendorf microcentrifuge and the supernatant collected. This latter was subsequently diluted and filtered prior to injection as described above.

RESULTS AND DISCUSSION

Fig. 1 depicts the structures of CAP (1-*p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol, molecular mass 323.1) and the following four analogues that are presumed to be metabolic products of the drug: DH-CAP (4′-nitro-2-dichloroacetamido-3-hydroxypropiophenone, molecular mass 321.1). NPAP [(*R*)-4′-nitro-2-amino-3-hydroxypropiophenone, hydrochloride, molecular mass 246.7], NO-CAP (1-*p*-nitrosophenyl-2-dichloroacetamido-1,3-propanediol, molecular mass

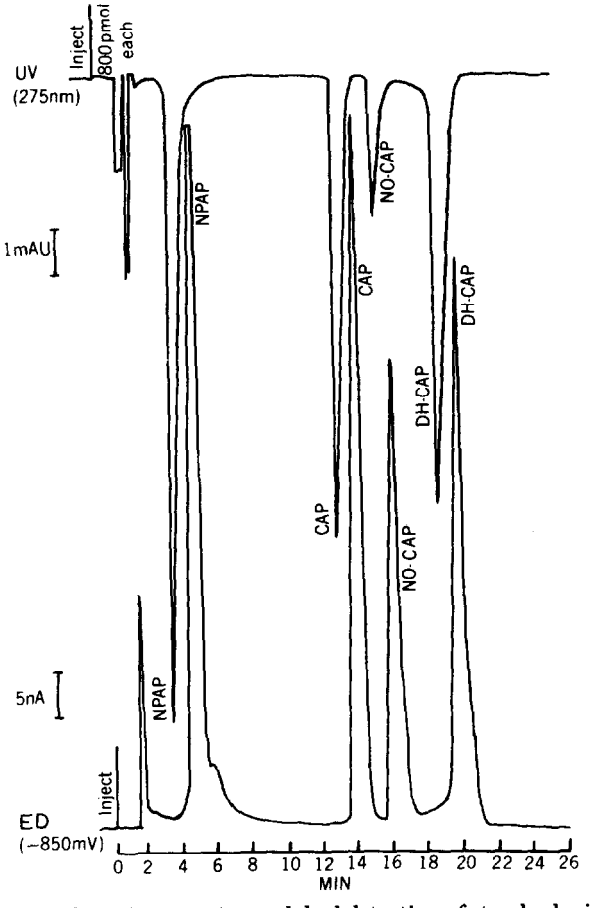


Fig. 2. HPLC separation and dual detection of standard mixture of NPAP, CAP, NO-CAP and DH-CAP.

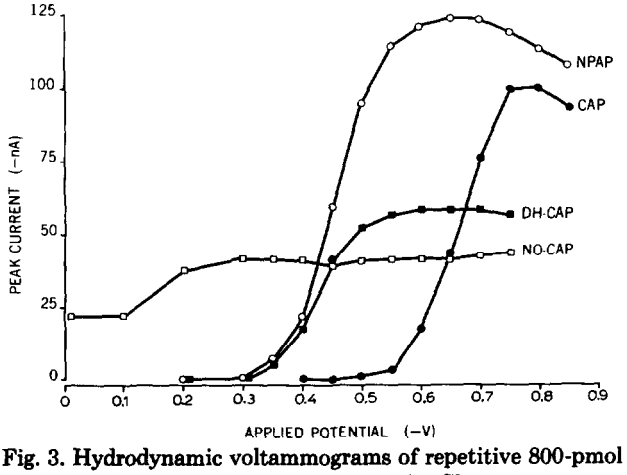
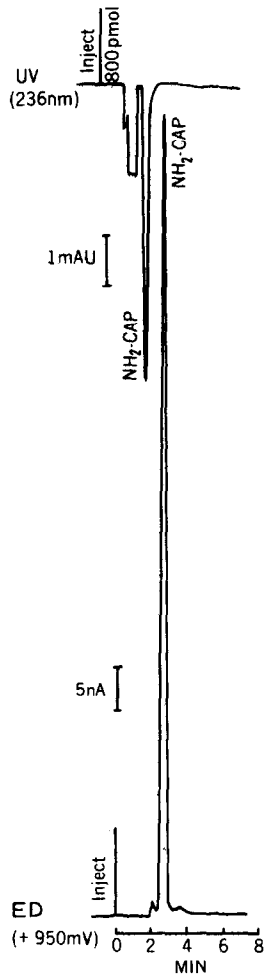


Fig. 3. Hydrodynamic voltammograms of repetitive 800-pmol injections of NPAP, CAP, NO-CAP and DH-CAP. All potentials were vs. Ag/AgCl.



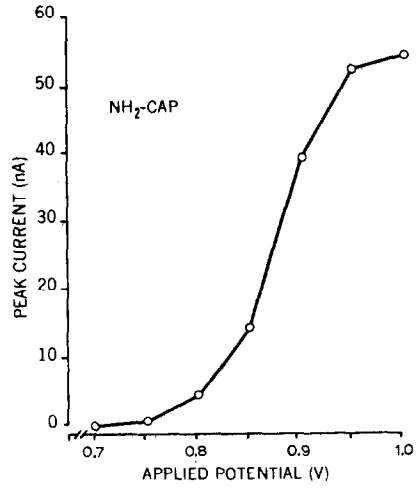


Fig. 4. In tandem HPLC chromatograms of NH_2-CAP as detected by UV and electrochemical detectors.

Fig. 5. Hydrodynamic voltammograms of repetitive 400-pmol injections of NH_2-CAP. All potentials were vs. Ag/AgCl.

307.1) and NH_2-CAP (1-*p*-aminophenyl-2-dichloroacetamido-1,3-propanediol, molecular mass 293.1).

A typical dual UV and ED chromatogram of a CAP, DH-CAP, NPAP and NO-CAP mixture is shown in Fig. 2. Detection was accomplished with the UV detector set at 275 nm and the ED detector at −850 mV. Sensitivity of detection for these compounds was in the picomole range with the UV mode, while it was possible to reach detection in the subpicomole range with the reductive ED mode (the electrochemical detector response, tested between 1 and 800 pmol, was linear). Solvent front peaks appeared between 1 and 2 min after injection in both modes. A residual oxygen peak was also detected at 5.87 min.

Hydrodynamic voltammograms of CAP, DH-CAP, NPAP and NO-CAP were

determined by repetitive injections of these compounds on our reversed-phase column followed by recording the responses of peak current for each applied electrode potential (Fig. 3). No currents were detected with CAP, DH-CAP and NPAP until the potential was raised to about −0.5, −0.35 and −0.35 V, respectively, whereas with NO-CAP significant current was detected at a potential as low as −0.005 V. The currents continued to rise until −0.75, −0.65, −0.55 and −0.20 V were applied with CAP, NPAP, DH-CAP and NO-CAP, respectively; then they leveled off to yield either a constant response (e.g. DH-CAP and NPAP) or a slight decrease with higher potential (e.g. CAP and NPAP). Accordingly, the optimum operating potential to select with a mixture of these compounds is −0.75 V.

Dual UV and oxidative ED of NH_2-CAP is illustrated in Fig. 4. Detection was done with the UV detector set at 235 nm (the absorption maximum 216 nm of this compound could not be used with our mobile phase) and the electrochemical detector at +0.95 V. A broad solvent front peak was detected in UV, whereas the oxidative ED chromatogram was free of such a peak.

Fig. 5 shows the hydrodynamic voltammogram of NH_2-CAP. Current responses started at ca. +0.75 V and continued to rise to ca. +1.0 V where a plateau is reached. Under our conditions, the optimum operating potential for this compound is +0.95 V.

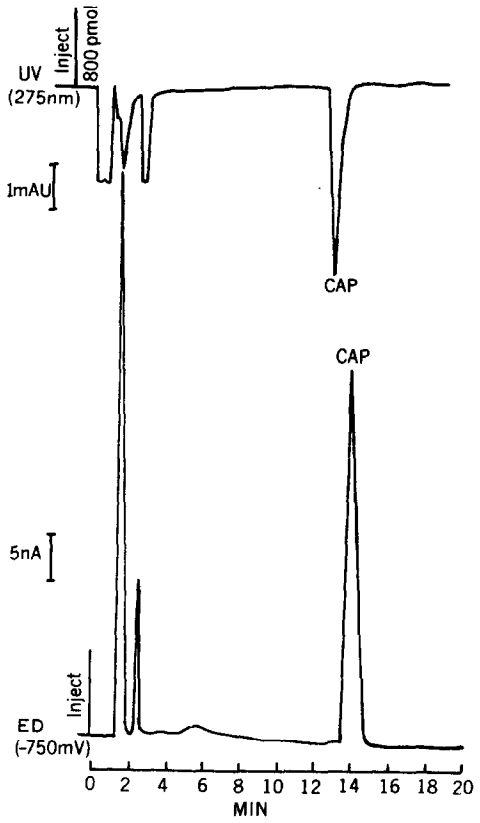


Fig. 6. Separation and dual detection of CAP after 60 min incubation with human blood.

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS AND DETECTION PARAMETERS OF CAP AND FOUR ANALOGUES

Based on conditions described under Experimental. RRT = relative retention time computed in relation to CAP which had a retention time of 13.77 min (Fig. 2). UV λ_{max} = ultraviolet absorption maximum as determined by spectrophotometric measurements. $ED_{red\ max}$ = maximum applied potential needed to obtain highest peak current by reductive electrochemical measurements. $ED_{ox\ max}$ = maximum applied potential needed to obtain highest peak current by oxidative electrochemical measurements.

Compound	RRT	UV λ_{max} (nm)	$ED_{red\ max}$ (mV)	$ED_{ox\ max}$ (V)
CAP	1.00	280	−750	N.C.★
NPAP	0.33	266	−650	N.C.
NO-CAP	1.16	315	−200	N.C.
DH-CAP	1.43	266	−550	N.C.
NH_2-CAP	N.A.★★	216	N.C.	+1.0

★N.C. = no current detected with applied potential up to 950 mV.

★★RRT not computed for NH_2-CAP. The compound was not detected, neither by ED reductive mode nor by UV at 275 nm. Under the conditions of Fig. 4, NH_2-CAP had a retention time of 2.51 min.

Applicability of the procedure to biological fluids is shown in Fig. 6 where CAP was incubated with human blood for 60 min as described under Experimental. After stopping the incubation by perchloric acid, the denatured proteins were removed by centrifugation and then filtration. Although the use of acid to stop the reaction results in precipitating part of the CAP with the denatured protein (0- and 60-min incubation assays gave similar chromatograms), the HPLC separation and dual UV–ED of CAP derived from blood was accomplished as anticipated (Fig. 6).

Table I summarizes the chromatographic characteristics and detection parameters of CAP, NPAP, NO-CAP, DH-CAP and NH_2-CAP. Relative retention times were computed for compounds detected by reductive ED. The data in Table I provide basis for the use of peak-height ratios for the identification of unknown metabolites.

The dual detection system described in this paper provides a highly sensitive and selective analytical method. In addition to its capacity of separating, quantitating and identifying CAP and its analogues, it provides a long-time awaited tool to uncover some of the important compounds produced by the metabolism of either CAP or other nitro aromatic compounds.

ACKNOWLEDGEMENT

This work was supported in part by NIH Grant AM 26218.

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