Journal of Chromatography, 162 (1979) 480—484
Biomedical Applications

CHROMBIO. 259

Note

Determination of serum chloramphenicol by high-performance liquid chromatography

J. CRECHIOLO and R.E. HILL

Department of Pathology, McMaster University, Hamilton, Ontario (Canada)

(First received May 17th, 1978; revised manuscript received August 11th, 1978)

Chloramphenicol, D(—)-*threo*-2,2-dichloro-N-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenethyl] acetamide is a broad spectrum antibiotic with bacteriostatic action. It is known to be highly toxic against the erythroid function of the bone marrow [1]. In premature infants, who lack an effective glucuronidation pathway, normal therapeutic doses can accumulate leading to fatal toxicity [2]. Despite this, it is the drug of choice in some situations, and paradoxically, its effectiveness against *H. influenzae* meningitis has lead to an increase in its use in infant patients who are, in turn, most susceptible to life-threatening toxicity from the drug. Under such circumstances it is clear that knowledge of the drug concentration in blood should permit optimum usage without fear of serious toxicity and that the availability of a rapid, accurate assay procedure would be an important adjunct to safe therapy.

Several techniques for the assay of chloramphenicol have been reported [3]. Of these, gas chromatographic procedures have been applied successfully to its analysis in serum [4, 5]. However, this approach requires prior derivatization to a silyl derivative and it is known that multiple peaks due to mono-, di- and trisilyl derivatives can ensue unless appropriate reagents and solvents are used [6]. High-performance liquid chromatography (HPLC) has been used to analyse chloramphenicol during its commercial production [7, 8], and recently has been applied to serum analysis [9]. This approach [9] is sensitive but requires a relatively large sample volume (500 μl) and a two-step extraction procedure. An internal standard was not included and accurate quantitation of unknowns must rest upon careful volume manipulations which can be tedious and error prone in the routine setting. It is noteworthy also that the

authors monitor the column effluent at 254 nm rather than 280 nm (λ_{max} chloramphenicol = 280 nm [10], and report the capacity factor, k', of 0 for thiamphenicol on a C_{18} reversed phase in a water methanol (70:30, v/v) system. We now report an HPLC assay specifically designed for routine analysis, which requires a single extraction step from a small volume of serum (50 µl) followed by reversed-phase chromatography. Thiamphenicol (D-*threo*-2,2-dichloro-N-[β-hydroxy-α-(hydroxymethyl)-*p*-(methylsulphonlyl) phenethyl] acetamide), a closely related antibiotic not used in clinical practice in North America serves as the internal standard.

EXPERIMENTAL

Apparatus

The liquid chromatogram used was an ALC Model 202, with Model 6000A pump, U6K injector and Model 440 absorbance detector (Waters Assoc., Milford, Mass., U.S.A.).

Chromatographic Conditions

Reversed-phase chromatography using two types of bonded phase was investigated and two equally appropriate systems arrived at.

System 1. A stainless-steel column (30 cm × 4 mm I.D.) packed with a stable reversed-phase stationary phase consisting of porous silica beads (mean diameter 10 µm) coated with a chemically bonded monolayer of octadecylsilane (µBondapack C_{18}, Waters Assoc.). The mobile phase is methanol—water (40:60, v/v) with a flow-rate of 1.7 ml/min and an operating pressure of 17.25 MPa (2500 p.s.i.).

System 2. A column of identical dimensions with a chemically bonded monolayer of cyanopropylsilane (µBondapack CN, Waters Assoc.). The mobile phase is methanol—water (20:80, v/v) with a flow-rate of 2.0 ml/min and an operating pressure of 17.25 MPa (2500 p.s.i.).

In both systems the operating temperature is ambient. The column effluent is monitored continuously at 280 nm with a full scale deflection of 0.05 A. A short methanol wash (20 ml at 1 ml/min) at the end of each analytical day removes strongly retained solutes from both phases.

Reagents

All chemicals are reagent grade. Chloramphenicol was donated by Parke Davis, Ontario, Canada. Thiamphenicol was purchased from Sigma (St. Louis, Mo., U.S.A.). Solvents are routinely filtered through 0.45-µm filters (Millipore Corp., Bedford, Mass., U.S.A.) prior to use in the liquid chromatograph.

Standards

Chloramphenicol (40 mg) is dissolved in methanol (10 ml). A 2-ml sample of this solution is diluted to 100 ml with plasma. This standard (80 mg/l) is serially diluted with plasma to prepare standards containing 60, 40, 20 and 10 mg/l, respectively. These preparations are divided into small aliquots (ca. 0.5 ml) and frozen (—20°). The internal standard, thiamphenicol (20 mg) is dissolved in ethyl acetate (1 l) and this solution serves as the extraction solvent

Extraction

Serum or plasma (50 μl) is added to a 50-ml glass tube fitted with a PTFE-lined screw cap. Ethyl acetate (5 ml), containing the internal standard is added, followed by sodium chloride (ca. 1 g). Extraction is for 10 min (Buchler Omnishaker), followed by centrifugation at 500 *g* for 2 min. A portion of the organic layer (ca. 4 ml) is transferred into a disposable tube and taken to dryness by warming under a stream of dry nitrogen. The residue is dissolved in methanol (ca. 40 μl) and 25 μl is injected into the chromatograph. This procedure is followed for patient and standard samples. Standard curves are constructed by plotting the peak height ratios of chloramphenicol to thiamphenicol against the chloramphenicol concentration in each standard. The level of chloramphenicol in an unknown sample is derived from this curve.

RESULTS AND DISCUSSION

Fig. 1 is a chromatogram of a 20 mg/l plasma standard on the C_{18} phase. In

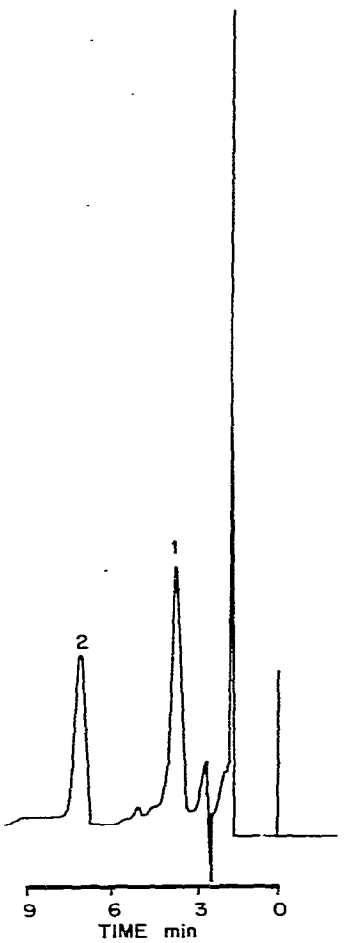
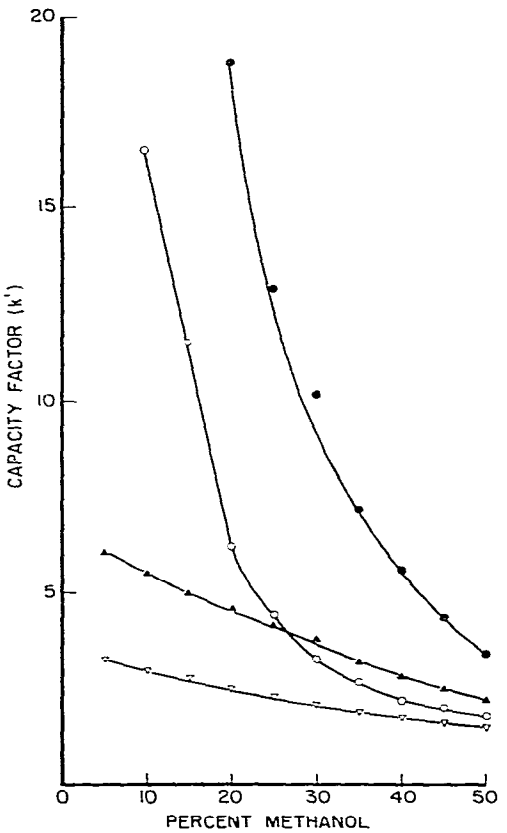


Fig. 1. Chromatogram of a plasma extract (20 mg/l) on the C_{18} phase. 1 = Thiamphenicol; 2 = chloramphenicol.

this case and on the —CN phase the chromatography is complete within 10 min with baseline separation between the two solutes. The k' values for chloramphenicol and thiamphenicol in the C_{18} system are 5.6 and 2.2 and in the —CN system 4.6 and 2.5, respectively (these data for thiamphenicol contrast with the $k' = 0$ reported by Wal et al. [9]. This illustrates the potentially wide variance in behaviour between bonded reversed-phase columns from different sources). The mechanisms of solute retention by —CN phases have not been investigated as fully as those for —C_{18} alkyl phases. However, under the conditions employed herein, the —C_2H_4CN function obeys "rule of thumb" reversed-phase behaviour and could be viewed as a short alkyl chain phase. In both systems the capacity factors of both solutes are reduced by increasing the methanol concentration of the solvent (Fig. 2) and it was by adjusting this component, together with flow-rate, that the optimum chromatographic conditions were achieved. Also the C_{18} phase exhibits much greater solute retention (Fig. 2) than the —C_2H_4CN phase and this behaviour is in accordance with many observations in reversed-phase chromatography [11—14], which demon-



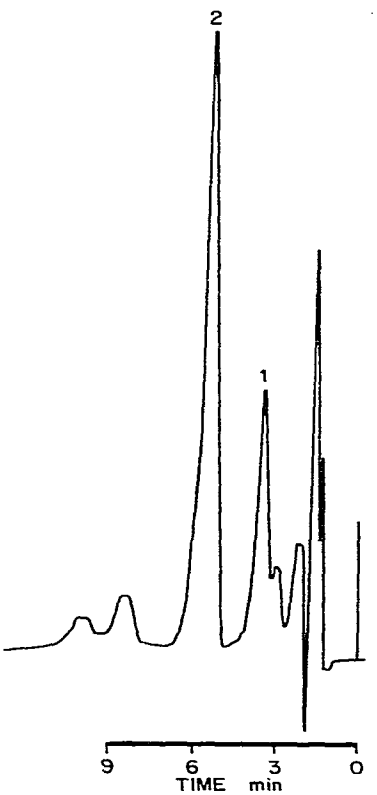


Fig. 2. Plot of k' vs. percent methanol. •, Chloramphenicol; ○, thiamphenicol, C_{18} phase; ▲, chloramphenicol; △, thiamphenicol, —CN phase.

Fig. 3. Chromatogram of patient plasma extract on the —CN phase. 1 = Thiamphenicol; 2 = chloramphenicol.

strate that k' values increase as the alkyl chain length and percentage carbon content of the bonded reversed phases increase.

Fig. 3 shows a chromatogram on the —CN phase from a patient on chloramphenicol therapy with found plasma levels of 76.0 ± 6.5 (1 S.D.) μg/ml (C_{18} approach) and 75.5 ± 5.7 (1 S.D.) μg/ml (—CN approach), this patient was an adult receiving large doses of chloramphenicol for disseminated intravascular coagulation caused by systemic bacterial infection. A level of about 15 μg/ml in plasma is considered effective against the majority of sensitive organisms, whereas anemia from the effect of the drug on bone marrow occurs regularly when levels of 25 μg/ml or higher are experienced [15]. Analysis of the standards and plasma blank by both approaches showed the relationship between the plasma concentration and peak height ratio of chloramphenicol to thiamphenicol to be linear between 0 and 80 μg/ml. Typical regression equations for the standard curves are $y = -0.23 + 0.37x$, $r = 0.9986$ (C_{18}) and $y = 0.05 + 0.34x$, $r = 0.9994$ (—CN) (y = peak height ratio drug:internal standard and x = chloramphenicol concentration). The limit of detection is 2 μg/ml for each method. A pool sample containing chloramphenicol (30 μg/ml) was processed to determine the accuracy and precision of the methods. The between batch variations are 8.6%, mean = 31.7 ± 2.7 (1 S.D.) (C_{18}, $n = 30$) and 7.5%, mean = 31.5 ± 2.4 (1 S.D.) (—CN, $n = 30$).

The above data demonstrate that each approach possesses the linearity, limits of detection, precision and accuracy acceptable for a routine assay aimed at monitoring blood levels of chloramphenicol. The sample work-up and fast analysis time allow regular analysis on a routine basis and moreover, the small sample requirement (50 μl), which can be met by the capillary sampling technique, obviates the need for venipuncture and enhances analysis of infant and neonatal patients who are particularly susceptible to harmful overdose from this drug.

REFERENCES

1 R.O. Wallerstein, P.K. Condit, C.K. Kasper, J.W. Brown and F.R. Morrison, J.Amer. Med. Ass., 208 (1969) 2045.
2 C.F. Weiss, A.J. Glazko and J.K. Weston, N. Engl. J. Med., 262 (1960) 787.
3 D. Szulczewski and F. Eng, in K. Florey (Editor), Analytical Profiles of Drug Substances, Academic Press, New York, 1975, p. 47.
4 C.J. Least, N.J. Wiegand, G.F. Johnson and H.M. Solomon, Clin. Chem., 23 (1977) 220.
5 G.L. Resnick, D. Corbin and D.H. Sandberg, Anal. Chem., 38 (1966) 582.
6 G. Janssen and H. Vanderhaeghe, J. Chromatogr., 82 (1973) 297.
7 Gy. Vigh and J. Inczédy, J. Chromatogr., 102 (1974) 381.
8 Gy. Vigh and J. Inczédy, J. Chromatogr., 116 (1976) 472.
9 J.M. Wal, J.C. Peleran and G. Bories, J. Chromatogr., 145 (1978) 502.
10 I. Sunshine (Editor), Handbook of Analytical Toxicology, The Chemical Rubber Company, Cleveland, Ohio, 1969, p. 257.
11 R.E. Majors and M.J. Hopper, J. Chromatogr. Sci., 12 (1974) 767.
12 R.K. Gilpin, J.A. Korpi and C.A. Janicki, Anal. Chem., 47 (1975) 1498.
13 E.J. Kikta and E. Grushka, Anal. Chem., 48 (1976) 1098.
14 H. Hemetsberger, M. Kellermann and H. Ricken, Chromatographia, 10 (1977) 726.
15 L. Weinstein, in L.S. Goodman and A. Gilman (Editors), The Pharmacological Basis of Therapeutics, Collier-MacMillan, London, 1970, p. 1273.