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Note

Reversed-phase high-performance liquid chromatographic determination of chloramphenicol in small biological samples

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Despite the availability of newer antimicrobial agents, chloramphenicol (CAMP) is still widely used in the treatment of a number of serious infections, and is particularly valuable in the treatment of central infections when these occur in neonates. However, recommended dosage schedules that are now generally accepted were calculated from pharmacokinetic data which were derived from following the use of non-specific assay methods [1–3], and considerable variation has been reported in the dosage of CAMP used in the treatment of neonates and infants [4,5] although more recent studies using high-performance liquid chromatography (HPLC) have helped in modifying previous guidelines[6]. The drug can cause severe, even life-threatening adverse reactions, particularly in premature neonates, and these effects are frequently related to the accumulation of CAMP, which results in abnormally high plasma concentrations.

Tailoring the dose to be both safe and effective is difficult, especially in premature neonates whose capacity to metabolise and excrete the drug is both variable and unpredictable. Therefore, when CAMP, which might produce severe toxicity, is administered to treat a life-threatening infection where underdosage would also be disastrous, the determination of drug levels in plasma becomes a mandatory part of the treatment in order that the dose may be individualised. Not only must the assay service be readily available, but also the analytical method should be specific, accurate, precise, and capable of producing with only a tiny blood sample a result within a reasonable time.

Microbiological plate assay is still the most widely used method of analysing CAMP in biological fluids, but many alternative techniques have been reported, *e.g.*, enzymological[7], gas chromatographic[8] and bioluminescence[9] methods. Several HPLC procedures have been reported[10–22], and this is undoubtably the method of choice for CAMP assay. When intravenous (i.v.) administration is necessary, CAMP is given as its prodrug, the inactive succinate ester, and many methods emphasise their utility in measuring the ester in addition to the microbiologically active derivative. However, at the present time, there is no clinical justification for measuring the succinate in most patients. Many of the earlier HPLC methods have not been properly validated, some omit internal standards[10,11] and thus require accurate volume transfers, others use a large sample[10], or non-ambient conditions[11,12]. One did not even show any chromatograms[13].

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More recently, Aravind *et al.*[18] used the preferred approach of separating CAMP from the biological sample matrix by solvent extraction. Mildly acidic conditions were chosen for extraction and the authors did not discuss potential interference from other drugs. Nahata and Powell[19] also failed to assess interference from other drugs and prepared the samples for analysis by only precipitating proteins with acetonitrile. A similar sample pretreatment was used by Danzer[20], who assessed interferences and extended the procedure to simultaneously determine the cephalosporins, but the method requires two detectors in series. Kushida *et al.*[21] included CAMP in a procedure for the simultaneous determination of anticonvulsant drugs, but again failed to assess interferences adequately. More recently Ryan *et al.*[22] thoroughly validated a method that employed a phase separation extraction prior to chromatography.

This paper reports a simple, rapid, validated, reversed-phase HPLC assay for CAMP, which has been used to provide a Supraregional Service in the U.K. for the last five years.

EXPERIMENTAL

Chemicals and reagents

CAMP and its succinate ester were supplied by Parke-Davis (Eastleigh, U.K.). Methoxycarbamazepine was supplied by Ciba-Geigy (Horsham, U.K.). Tris(hydroxymethyl)aminomethane, dichloroethane, sodium hydroxide and acetic acid (Analar grades) were purchased from BDH (Poole, U.K.). Acetonitrile (S grade) was purchased from Rathburn Chemicals (Walkerburn, U.K.).

Chromatographic equipment and conditions

The reversed-phase HPLC system consisted of a Model M45 pump (Waters Assoc., Harrow, U.K.), Rheodyne 7125 injection valve (Magnus Scientific, Sandbach, U.K.) 5 cm × 4.4 mm I.D. Co:PELL ODS precolumn (Whatman, Maidstone, U.K.), 5-μm Hypersil MOS (C_8) column, 25 cm × 4.4 mm I.D. (Technicol, Stockport, U.K.), a Model 212 variable-wavelength detector (Cecil Instruments, Cambridge, U.K.) and a Model 28000 recorder (Bryans Southern, Mitcham, U.K.).

The mobile phase which was pumped at 1.8 ml/min, consisted of acetonitrile–acetate buffer (pH 5.8) (2:3). The buffer was prepared by adding 50 ml 1 *M* sodium hydroxide to 58 ml of 1 *M* acetic acid and diluting to 1.0 l with distilled water. Before use, the mobile phase was degassed by passing it through a Millipore 47-μm filter (Waters Assoc.). Chromatography was performed at room temperature. The detector sensitivity was set 0.2 a.u.f.s., and the wavelength at 280 mm.

Standards

A stock solution, containing 1 mg/ml CAMP, was prepared in ethanol. Working calibration standards (2.5–20 mg/l) were prepared by dispensing, with a Hamilton repeating syringe (Phase Separations, Queensferry, U.K.), varying amounts (25–200 μl) of this stock solution into 10-ml volumetric flasks which were then filled with equine plasma. After thorough mixing 2-ml aliquots of these were stored at −20°C. working standards were kept at 4°C. Quality control samples (5 and 25 mg/l) were prepared in a similar manner, but from an independent stock solution. A full set of

plasma calibrators and bilevel quality control samples were extracted through the procedure with each batch of specimens to be analysed.

Assay procedure

To 200 μl plasma standard or patient sample in a 10-ml conical centrifuge tube were added 400 μl Tris buffer (0.8 *M*), 50 μl internal standard (0.05 g/l methoxycarbamazepine in ethanol), and 7 ml dichloroethane. The tubes were Vortex mixed for 1 min and then left to stand for a few minutes while the phases separated. The upper, aqueous layer was discarded and the cloudy bottom phase was filtered (Whatman No. 1), and the dichloroethane was collected in a 10-ml conical tube. The solvent was evaporated to dryness at 50°C under a stream of air, and the residue was reconstituted in 100 μl of mobile phase prior to injecting 10–20 μl into the HPLC system. When less than 200 μl of sample is available from a patient, the procedure can readily be scaled down.

Quantification

After injecting the extracts from the plasma working standards into the column, the peak heights of CAMP and internal standard were measured from an extrapolated baseline. A calibration graph was prepared by plotting the ratio of the peak height of drug to peak height of internal standard against the CAMP concentration. The CAMP concentration in a sample was calculated by comparing the peak height ratio of the sample directly with the standard calibration graph.

Precision, accuracy and specificity

Between-run precision was evaluated from the results of the high and low plasma quality control samples that were analysed with each batch of samples. Accuracy was assessed by participating in a national quality assessment scheme, which circulates plasma samples, spiked with CAMP, on a monthly basis. Specificity was evaluated by analysing samples from patients receiving a wide variety of other drugs that are commonly prescribed with CAMP.

Method comparison

Residual samples from the routine service were frozen (−20°C) immediately after HPLC analysis had been completed and were later again analysed by either a microbiological plate assay or a bioluminescence assay. After six months storage many of the samples were analysed again by HPLC in order to establish whether chloramphenicol and the succinate ester were stable under the conditions used.

RESULTS AND DISCUSSION

Typical chromatograms are shown in Fig. 1. Tracing A is from an extract of a blank plasma. It demonstrates that no endogenous plasma components interfere with the assay and that the analysis time is short, the internal standard being eluted after only 2.2 min. Tracing B is from a 20-mg/l calibration standard extract. It shows sharp symmetrical peaks, allowing the use of peak heights rather than peak areas for quantitation. Tracing C is from a patient sample. It shows an additional peak, eluted after 1 min. The peak is frequently present in samples from patients

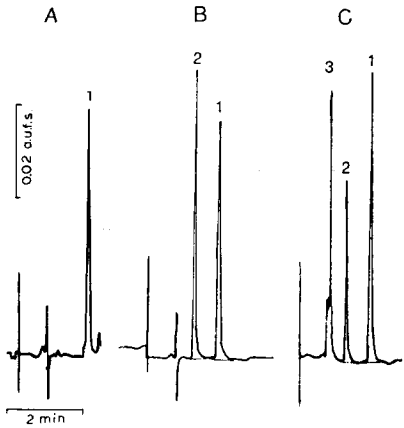

Fig. 1. Typical chromatogram of (A) a blank sample containing the internal standard, (B) 20 mg/l calibration standard, and (C) patient sample. Peaks: 1 = internal standard (methoxycarbamazepine), 2 = chloramphenicaol, and 3 = chloramphenicol succinate.

undergoing i.v. treatment with CAMP, and its retention time is identical to that of the succinate.

The peak height ratio of CAMP:methoxycarbamazepine from extracted plasma standards was rectilinear over the range 2.5–20 mg/l, and the analysis of samples having higher concentrations by processing a smaller aliquot indicated that the calibration graph extended to 60 mg/l. Since quantification was achieved by reference to plasma standards which were spiked with CAMP prior to being carried through the procedure it was not necessary to apply a recovery correction factor to the samples, therefore the absolute recovery was not determined. The limit of accurate measurement was 0.5 mg/l, and the between-run coefficient of variation of the method was 3.6 and 4.4% at 5 and 25 mg/l, respectively. A good correlation ($r = 0.9894$, $n = 31$) between the actual value of CAMP and that determined by HPLC was obtained in spiked samples circulated by the Southmead Microbiological Quality Assessment Scheme. The specificity of the method is excellent, and no endogenous compounds or drugs commonly prescribed with CAMP interfere with the method. The following drugs were tested: acyclovir, amikacin, ampicillin, benzylpenicillin, caffeine, carbenicillin, cefotaxime, cefuroxime, cimetidine, erythomycin, frusemide, fusidic acid, gentamicin, ketoconazole, metronidazole, miconazole, netilmicin, penicillin, phenobarbitone, phenytoin, sulfadimidine, sulphamethoxazole, theophylline, trimethoprim and vancomycin. Comparison of freshly prepared calibration standards with those kept at −20°C for six months showed that CAMP was stable under these conditions. Also, analysis of patients samples containing CAMP and the succinate both before and after storage for six months established that degradation of succinate to CAMP did not occur at −20°C. The employment of solvent extraction under mildly basic conditions, and the use of a long wavelength for monitoring the column effluent both help to enhance the specificity of the method. Solvent extraction eliminates hydrophilic material and mildly basic conditions preclude acid drugs from the final residue while ensuring that hydrolysis of succinate to CAMP does not occur. Pre-extraction of the drug by solvent from the biological sample, together with the

use of a short, easily replenished pre-column also extend the life of the analytical column, which remains efficient after more than 1000 injections.

The standard and quality control extracts are stable for several days, and by processing a relatively large volume of these there is sufficient residue to inject several times into the chromatograph. By setting up a number of spare tubes with each calibration run it is thus possible to analyse urgent single samples in approximately 30 min with minimal effort.

Correlation of the present HPLC assay with the various alternative methods was good: (1) microbiological plate assay, $n = 30$, $r = 0.9865$; (2) bioluminescence assay, $n = 45$, $r = 0.96$.

In conclusion, the analysis of CAMP by the reversed-phase HPLC method which incorporates a simple solvent extraction to separate the drug from the biological sample and methoxycarbamazepine as internal standard, is rapid, sensitive, specific, and reproducible. It has proved an excellent basis on which to build a service to assist with individualising CAMP therapy and has been used to analyse 3000 clinical samples during the last five years.

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