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Pharmaceutical analysis of 8-chloroadenosine 3′,5′-monophosphate

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Abstract: 8-Chloroadenosine 3′,5′-monophosphate (8-Cl-cAMP) has recently entered clinical trials as a new anticancer drug. 8-Cl-cAMP can undergo metabolism/degradation to nucleotides (8-Cl-AMP; 8-Cl-IMP), nucleosides (8-Cl-adenosine; 8-Cl-inosine) and bases (8-Cl-adenine; 8-Cl-xanthine and 8-Cl-hypoxanthine). A reversed-phase, gradient elution high-performance liquid chromatographic method is described which can resolve all the above from their non-chlorinated naturally occurring counterparts and 8-Bromo-cAMP. The assay was then utilized to perform pharmaceutical evaluations on the 8-Cl-cAMP formulation. Sterilization by filtration (0.22 μm pore size) resulted in no loss of material. Stability studies carried out in the cartridges used to continuously infuse (for 7 days) the drug to patients showed no significant degradation over 17 days. Purity determinations revealed the presence of up to nine impurities (related to adenine) and yielded a purity figure of 95.9–99.3% with considerable batch to batch variation.

Keywords: *8-Cl-cAMP; gradient elution; reversed-phase HPLC; stability; purity.*

Introduction

8-Chloroadenosine 3′,5′-monophosphate (8-Cl-cAMP) is a site selective cAMP analogue that exhibits antiproliferative effects through a mechanism believed to involve modulation of intracellular levels of the two iso-forms of the cAMP binding regulatory subunit of protein kinase A [1, 2]. Alternatively, it has been proposed that cytotoxicity to tumour cells may be mediated via the 8-Cl-adenosine product of *in situ* derived metabolism [3, 4]. 8-Cl-cAMP is now progressing through early clinical evaluation as a potential new anticancer drug. In this report a high-performance liquid chromatographic (HPLC) method is described which is capable of resolving 8-Cl-cAMP from all its major 8-chloro-metabolites/degradation products (see Fig. 1) and their non-chlorinated, naturally occurring counterparts. A series of pharmaceutical analyses have been performed on the material used by hospital pharmacists to formulate the drug for administration to patients.

Experimental

Materials

Analytical standards of 8-Cl-cAMP, its major 8-chlorinated metabolites (see Fig. 1) and 8-Bromo-cAMP were all from BioLog Life Science Institute, Bremen, Germany. Analytical standards of cAMP and its major metabolites (analogous to Fig. 1) were all from the Sigma Chemical Co. (Poole, UK). Bulk non-formulated 8-Cl-cAMP for administration to patients was manufactured and received from Tonen Corporation, Tokyo, Japan. All other reagents and chemicals were of the highest grade commercially available and water was de-ionized and bi-distilled in a quartz glass still.

High performance liquid chromatography

Apparatus consisted of a Hewlett–Packard Model 1090 liquid chromatograph with a diode array detector (DAD) (Hewlett–Packard Analytical, Waldbron, Germany). The stationary phase consisted of a Spherisorb ODS-2 (25 cm × 4.6 mm i.d.) stainless steel analytical column and a Spherisorb ODS-2 (1 cm × 4.6 mm) stainless steel pre-column (supplied by Crawford Scientific, Strathaven, Scotland, UK). The mobile phase comprised of sodium phosphate (pH 3.5, 10 mM) as solvent A and methanol as solvent B. Gradient elution was employed at a flow rate of 0.75 ml min^{-1}, at 40°C, using the following linear programme: time zero, 5% solvent B; time 3 min, 8%

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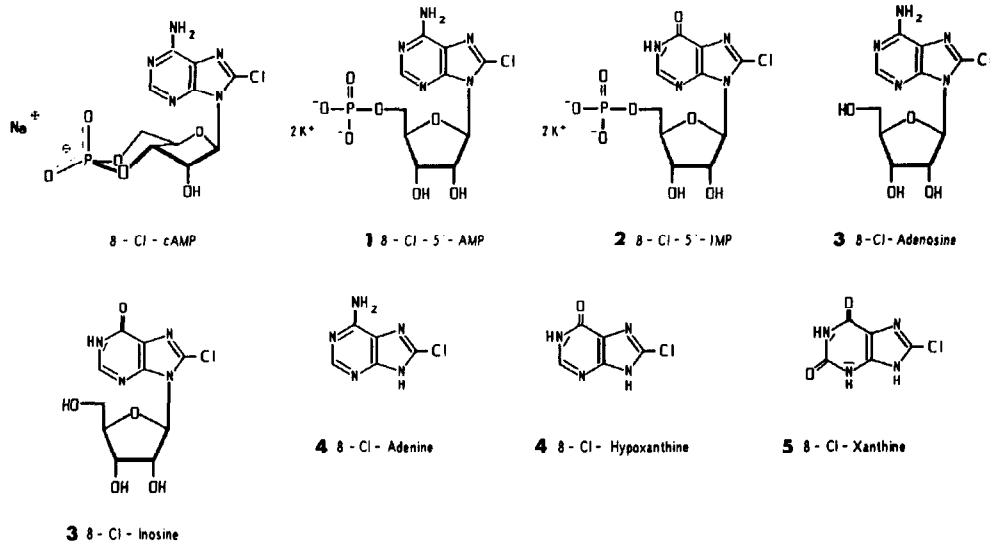

Figure 1
Products of metabolism/degradation of 8-Cl-cAMP

solvent B; time 15 min, 25% solvent B; time 20 min, 5% solvent B. The total run time was 25 min which allowed 5 min for complete re-equilibration of the mobile phase to occur (as determined spectrophotometrically using the DAD).

Pharmaceutical analysis

Ten ml of 0.1 and 1 mg ml^{-1} aqueous solutions of 8-Cl-cAMP were passed through a single use, non-pyrogenic, 0.22 μm pore size filter (Millipore, Molshein, France). These solutions were analysed (20 μl) by HPLC as above before and after filtration without further sample preparation.

Long term stability studies were executed in the infusion pump (CADD) cassette reservoirs (100 ml capacity, 76 cm extension line set) used to continuously administer drug to patients for 1 full week (Pharmacia Deltec Inc., St Paul, USA). 8-Cl-cAMP was reconstituted in sterile saline (0.9% sodium chloride). Cassettes were placed in the dark in a water bath set at 30°C (approximate external body temperature), to replicate as closely as possible the conditions they will be subjected to when worn by patients. Incubations were extended over 17 days. At each time point studied the first 3 ml withdrawn was discarded to eliminate material that had been stored in the extension line. A further 1 ml was withdrawn and this was directly analysed (20 μl) for 0.1 mg ml^{-1}, or first diluted in sterile water prior to analysis (20 μl) for 3 mg ml^{-1} and 6 mg ml^{-1}.

Purity determinations were carried out on four different batches of material. Samples were received from two different pharmacy sources: the Western General Hospital, Edinburgh, UK and the John Radcliffe Hospital, Oxford, UK (courtesy of Dr Nicola Stoner and Professor Adrian Harris). Analyses were performed on aqueous 1 mg ml^{-1} solutions. Before analysing one of these samples, the HPLC column was washed with methanol and a dummy run was performed with 20 μl of distilled water to establish a flat baseline.

Results and Discussion

High performance liquid chromatography

A typical chromatogram illustrating the separation of 8-Cl-cAMP and all other (16) components of interest is to be found in Fig. 2 and the chromatographic characteristics of the separation (including DAD absorption maxima) are contained in Table 1. With the exception of hypoxanthine and 8-Cl-AMP all other components were well resolved but, curiously, adenine and 8-Cl-adenine yielded poor peaks. Calibration curves were evaluated from a concentration of 0.1 μg ml^{-1} up to 3 mg ml^{-1}

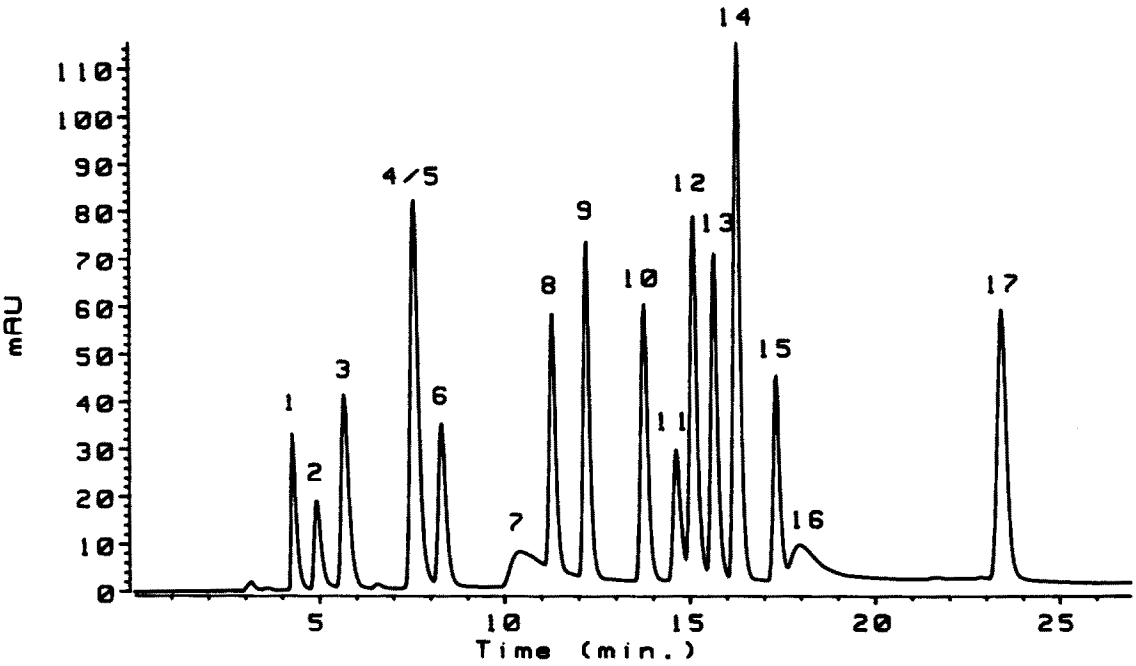

Figure 2
Separation of 8-Cl-cAMP and its major metabolites by reversed-phase HPLC. The stationary phase was Spherisorb ODS-2 and the mobile phase consisted of sodium phosphate (pH 3.5, 10 mM) as solvent A and methanol as solvent B. Gradient elution was employed by increasing solvent B according to the linear programme described in Experimental. Identity of all numbered components, their retention times, relative quantitation and U.V. absorption maxima are to be found in Table 1.

Table 1
HPLC with DAD of 8-Cl-cAMP and its metabolites

Component	Number (see Fig. 2)	Retention time (min)	CV (%)	Relative quantitation* (260 nm)	U.V. maxima (nm)
IMP	1	4.10	2.6	0.35	249
8-Cl-IMP	2	4.64	4.1	0.46	253
AMP	3	5.25	4.2	0.80	258
Hypoxanthine	4	6.91	4.3	0.85	249
8-Cl-AMP	5	6.91	4.3	0.40	261
Xanthine	6	7.47	5.8	0.85	268
Adenine	7	9.65	5.0	–	261
Inosine	8	10.34	3.9	0.65	249
cAMP	9	11.44	3.4	1.00	258
8-Cl-Hypoxanthine	10	12.77	3.5	0.88	253
8-Cl-Xanthine	11	13.65	3.3	0.87	274
Adenosine	12	14.25	2.5	1.00	260
8-Cl-cAMP	13	14.99	2.8	1.00	261
8-Br-cAMP	14	15.69	2.7	0.80	265
8-Cl-Inosine	15	16.53	2.2	0.65	253
8-Cl-Adenine	16	17.33	2.1	1.00	265
8-Cl-Adenosine	17	22.82	1.3	1.00	263

* Relative quantitation is defined as the ratio: peak area of component of interest/peak area of 8-Cl-cAMP (measured at equivalent concentrations, normally $\mu g\ ml^{-1}$).

for 8-Cl-cAMP and up to 1 mg ml^{-1} for all the metabolites, and these proved to be linear with regression correlation coefficients of 0.998 or better. Previous HPLC separations of 8-Cl-cAMP have concentrated on attempts to resolve either the nucleotide metabolites [5, 6] or the nucleoside metabolites of the drug, in particular 8-Cl-adenosine and 8-Cl-inosine [7, 8]. To the best of the authors' knowledge, the method presented in the work, represents the first attempt to resolve on one chromatogram all the major metabolites/degradation products of 8-Cl-cAMP including bases, nucleosides and nucleotides.

Table 2
Batch to batch purity determined by HPLC with DAD of 8-Cl-cAMP bulk material used to formulate the drug for administration to patients by continuous infusion over several weeks

Batch	Analyses (*n*)	% Purity (mean ± SD)	Impurities (*n*)	Profile (% ± SD)				
				cAMP	8-hydroxy-cAMP	8-Cl-adenine	8-Br-cAMP	Others*
1	6	98.2 ± 0.44	5	0.77 ± 0.04	0.18 ± 0.04	0.45 ± 0.05	—	0.47 ± 0.06
2	7	99.3 ± 0.29	3	0.28 ± 0.06	—	—	0.20 ± 0.02	0.11 ± 0.04
3	2	97.2	9	0.11	0.12	0.71	0.62	1.3
4	3	95.9 ± 0.06	9	0.24 ± 0.04	0.08 ± 0.01	0.58 ± 0.20	0.23 ± 0.10	2.95 ± 0.24†

*Others had U.V. absorption maxima at 261 nm and were quantitated relative to 8-Cl-cAMP using a factor of 1.0 (see Table 1).
†Others included one major impurity with a retention time of 18.0 min and absorption maximum of 261 nm and accounted for 2.4%.

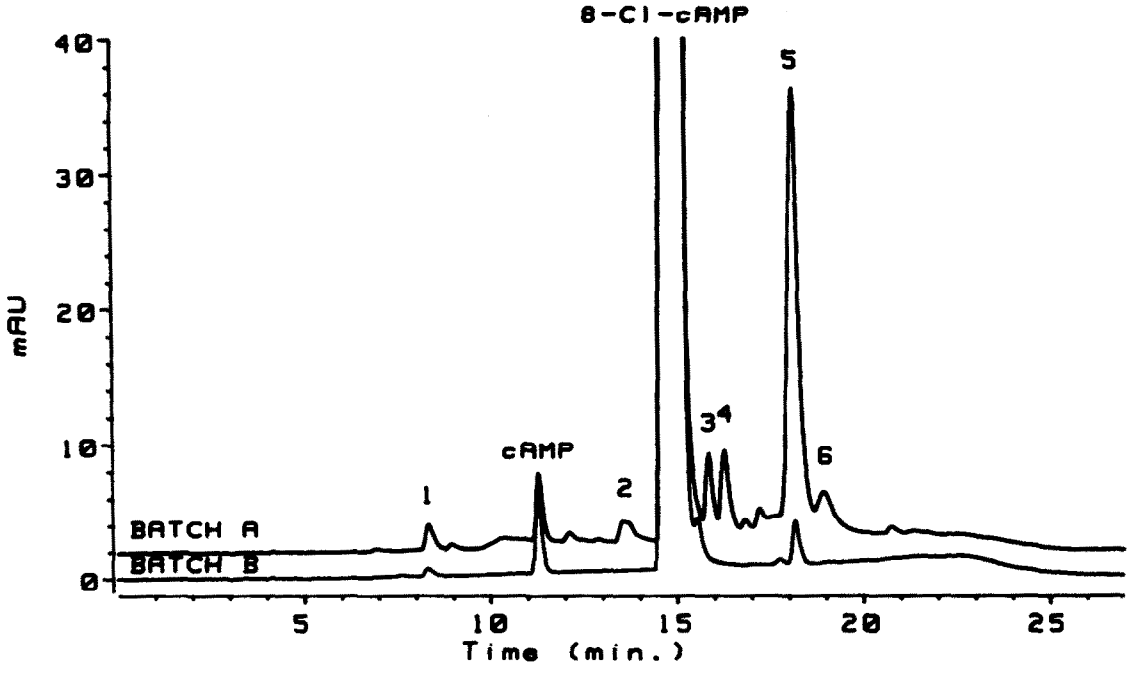

Figure 3
HPLC analysis of 8-Cl-cAMP bulk material supplied by the manufacturer to hospital pharmacists for formulation and administration to patients. Chromatogram BATCH A is an analysis of batch 4 from Table 2 and chromatogram BATCH B is an analysis of batch 2 from Table 2. Peak 1 is believed to be 8-hydroxy-cAMP; peak 3 is 8-Bromo-cAMP and peaks 2, 4, 5 and 6 remain unidentified.

Pharmaceutical analysis

Filtration of aqueous solutions of 8-Cl-cAMP resulted in no detectable loss of material (including impurities, see below), or no introduction of new peaks. Thus, filtration would appear to be a feasible method for on site sterilization of the drug.

Over an extended period of 17 days no significant (<5%) degradation of 8-Cl-cAMP (or impurities, see below) occurred in the infusion cassette used to administer the drug to patients. These cartridges are normally worn for 1 week.

Considerable batch to batch variation was recorded in the purity of the bulk material which ranged from 95.9 to 99.3% (see Table 2) and several impurities related to adenine were detected. Using HPLC with DAD 4 major impurities were identified as: cAMP, 8-hydroxy-cAMP, 8-Cl-adenine and 8-Br-cAMP. Although, in the case of 8-hydroxy-cAMP, this was not confirmed by reference to a standard. This profile is in good agreement with manufacturers' information. The complete purity profiles of the four batches analysed are contained in Table 2 and examples of chromatograms from the analysis of two different batches are shown in Fig. 3. At least five contaminants remain unidentified and these made up between 0.47 and 2.95% of the bulk material, including in one batch (batch 4, Table 2 and BATCH A, Fig. 3) one specific impurity (retention time, 18 min; U.V. absorption maxima, 261 nm) which accounted for 2.4% of bulk material.

The importance of identifying contaminants of 8-Cl-cAMP cannot be over stressed. Since the drug is administered to patients as a continuous infusion over a period of several weeks, even minor impurities may have the opportunity to accumulate and exert unwanted pharmacological or toxicological actions. Certain potential impurities, such as 8-Cl-adenosine, are highly cytotoxic (with IC_{50} values in the nM range) [9] and their presence will have implications for the way hospital pharmacists handle the material. The data presented in this work show that the purity of the bulk material sent to hospital pharmacists can vary considerably, although in no case was 8-Cl-adenosine detected. They suggest that each new batch should undergo quality control analysis prior to administration to patients. Also, it is recommended that further work is

carried out to identify all the impurities present in the pharmaceutical preparation of 8-Cl-cAMP.

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