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Short communication

Sensitive determination of 8-chloroadenosine 3′,5′-monophosphate and 8-chloroadenosine in plasma by high-performance liquid chromatography

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Abstract

8-Chloroadenosine 3′,5′-monophosphate (8-Cl-cAMP) is progressing through clinical evaluation as an anticancer drug. There is debate as to whether 8-Cl-cAMP is the active principal or its cytotoxic metabolite 8-Cl-adenosine. Separate high-performance liquid chromatographic methods are described for (i) 8-Cl-cAMP and its nucleotide metabolites (with 8-Br-cAMP as internal standard), and (ii) 8-Cl-adenosine. Both methods use a reversed-phase (Spherisorb ODS-2) stationary phase and a mobile phase consisting of sodium phosphate buffer (10 m*M*, pH 3.5) and methanol but with gradient elution for the nucleotides and isocratic elution for 8-Cl-adenosine. 8-Cl-cAMP and related nucleotides are extracted from plasma using strong anion-exchange solid-phase extraction (SPE) and 8-Cl-adenosine is extracted using reversed-phase (C_8) SPE. Both techniques enabled analyses to be performed at high detector sensitivity with minimal interference. Limit of detection in plasma was 10 ng/ml for both 8-Cl-cAMP and 8-Cl-adenosine. When applied to the analysis of plasma samples from a patient treated with a low dose continuous infusion of 25 μg/kg/h, steady-state concentrations centred around 60 ng/ml 8-Cl-cAMP were determined. In the same patient 8-Cl-adenosine was not detected. Application of this methodology will aid in the further development of 8-Cl-cAMP as a potential new form of anticancer treatment.

1. 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 is mediated via the 8-Cl-adenosine product of *in situ* derived metabolism and that 8-Cl-cAMP is, in its own right, inactive but merely serves as a pro-drug to release the active metabolite [3,4]. The subject of mechanism of action has aroused considerable debate [5,6]. 8-Cl-cAMP has recently entered clinical evaluation as an anticancer drug where it is administered as a continuous infusion at extremely low does (10–40 μg/kg/h). In order to perform pharmacokinetic studies in patients and address the issue of metabolism to cytotoxic species, sensitive analytical methodologies have been developed for 8-Cl-cAMP and 8-Cl-adenosine based on high-performance liquid chroma-

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tography (HPLC) and solid-phase extraction (SPE).

2. Experimental

2.1. Chemicals and drug standards

All methanol was HPLC reagent grade and was from Rathburn Chemicals (Walkerburn, UK). Sodium dihydrogen phosphate (Aristar grade) and orthophosphoric acid (AnalaR grade) were from BDH (Poole, UK). Tri-sodium citrate buffer was from Sigma (Poole, UK). Water was deionised and double distilled in a quartz glass still. All other chemicals were of the highest grade commercially available and were used as received. Analytical standards of 8-Cl-cAMP, 8-Br-cAMP, 8-Cl-adenosine, 8-Cl-AMP, 8-Cl-adenine, 8-Cl-IMP, 8-Cl-inosine, 8-Cl-hypoxanthine and 8-Cl-xanthine were all from BioLog Life Science Institute (Bremen, Germany). Analytical standards of the non-chlorinated naturally occurring counterparts of the above were obtained from Sigma. All analytical standards were dissolved in sterile distilled water (allowing for their greatly different aqueous solubilities), filtered and aliquoted and were stored at −40°C. New batches of standards were made up every month.

2.2. High-performance liquid chromatography

Apparatus consisted of a Hewlett-Packard Model 1090 liquid chromatograph with a diode-array detector (DAD, set at 260 nm) (Hewlett-Packard Analytical, Waldbron, Germany) configured as reported previously [7]. For the analysis of 8-Cl-cAMP, its nucleotide metabolites and 8-Br-cAMP conditions were as follows. 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 I.D.) stainless steel pre-column (supplied by Crawford Scientific, Strathaven, UK). The mobile phase consisted of sodium phosphate (pH 3.5, 10 m*M*) as buffer A and methanol as solvent B. Gradient elution was employed at a flow-rate of 0.75 ml/min, at 40°C, using the following linear programme: $t = 0$, 5% solvent B; $t = 3$ min, 8% solvent B; $t = 15$ min, 25% solvent B; $t = 20$ min, 5% solvent B; the total run time was 25 min. In the analysis of 8-Cl-adenosine conditions were identical to above except that elution was isocratic at 1 ml/min with sodium phosphate (pH 3.5, 10 m*M*)–methanol (77.5:22.5, v/v).

2.3. Solid-phase extraction

8-Cl-cAMP, its nucleotide metabolites (8-Cl-AMP, 8-Cl-IMP, cAMP, AMP and IMP) and 8-Br-cAMP were extracted from plasma using Bond Elut strong anion-exchange (SAX) mini-columns (500 mg sorbent, 3 ml reservoir capacity, supplied by Crawford Scientific) operating under negative pressure. Columns were first activated with 2 ml methanol, then washed with 2 ml water. A maximum volume of 1 ml of plasma was then loaded on to the columns, a larger volume of plasma resulted in greatly reduced recoveries. Subsequently, columns were washed twice with 2 ml of water and finally eluted with 1 ml of 0.2 *M* tri-sodium citrate. The citrate buffer eluate was filtered and up to 200 μl was injected onto the liquid chromatograph. 8-Cl-adenosine was extracted from plasma using Bond Elut C_8 reversed-phase mini-columns (100 mg sorbent, 1-ml reservoirs, Crawford Scientific). Columns were activated with 1 ml methanol and washed with 1 ml of water prior to loading 1 ml of plasma. They were then washed twice with 1 ml of water prior to elution with 0.5 ml of 10 m*M* sodium phosphate (pH 3.5)–methanol (70:30, v/v). The final eluent was filtered and 100 μl was analyzed by HPLC.

2.4. Analysis of patients samples

Plasma samples were obtained from a patient receiving 8-Cl-cAMP as a continuous infusion at a dose level of 25 μg/kg/h during a Phase I study conducted in the Western General Hospital, Edinburgh, UK. Blood specimens were collected in tubes containing EDTA to prevent *in situ* conversion of 8-Cl-cAMP to 8-Cl-AMP

(and finally 8-Cl-adenosine) through the action of plasma cAMP phosphodiesterase. Plasmas were thawed at 4°C and kept on ice prior to SPE which was normally performed immediately after samples had thawed. A batch of specimens were not thawed simultaneously but were analyzed sequentially.

3. Results

3.1. High-performance liquid chromatography

Table 1 contains the chromatographic characteristics of the separations developed and Fig. 1 (chromatogram C) illustrates an example of the separation of a standard mixture (20 ng for each compound) of the 7 nucleotides of interest. It should be noted that baseline resolution is achieved in each case without any loss of peak sharpness and this is reflected by detection limits (monitoring at 260 nm) in the low nanogram range (Table 1). A chromatogram of 8-Cl-adenosine is shown in Fig. 2 (trace C). Non-extracted standard curves for all 8 components were linear over the concentration range 0.1–100 μg/ml (2–2000 ng on column) with regression correlation coefficients of 0.999 or better.

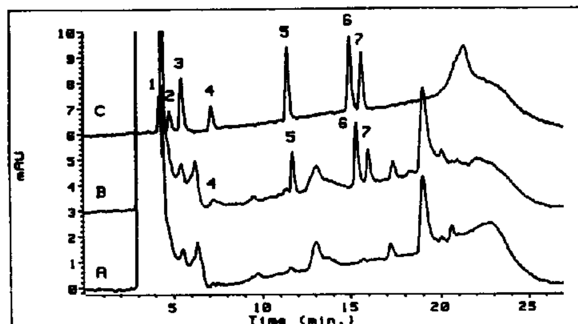

Fig. 1. Reversed-phase, gradient elution HPLC of 8-Cl-cAMP and its nucleotide metabolites. HPLC and SPE are described in Experimental. Chromatogram C is a mixture of standards with each component representing 20 ng. Peaks: 1 = IMP; 2 = 8-Cl-IMP; 3 = AMP; 4 = 8-Cl-AMP; 5 = cAMP; 6 = 8-Cl-cAMP, and 7 = 8-Br-cAMP. Chromatogram B is a plasma extract spiked with 20 ng of each component. Peaks are as above and note that the 4 mononucleotides are not recovered. Chromatogram A is a blank plasma extract.

3.2. Solid-phase extraction

It was found necessary to develop different sample preparation methods for the nucleotide and nucleoside components in the analysis of 8-Cl-cAMP and its metabolites: anion-exchange SPE for nucleotides and reversed phase (C_8) SPE for 8-Cl-adenosine. The bases 8-Cl-adenine, 8-Cl-hypoxanthine and 8-Cl-xanthine (and to a

Table 1
High-performance liquid chromatography of 8-Cl-cAMP, its nucleotide metabolites and 8-Cl-adenosine

Component	Retention time (mean ± C.V.) (min)	Detection limit		Absorption maxima (nm)
		On column (ng)	After extraction (ng/ml)	
IMP	4.18 ± 5.3	4	NE[a]	249
8-Cl-IMP	4.67 ± 2.7	4	NE	253
AMP	5.41 ± 4.5	2	NE	258
8-Cl-AMP	7.27 ± 5.8	4	NE	261
cAMP	11.73 ± 3.8	1	10	258
8-Cl-cAMP	15.36 ± 4.5	1	10	261
8-Br-cAMP	16.05 ± 4.4	2	10	265
8-Cl-adenosine	8.13 ± 0.3	1	10	263

[a] NE = not evaluable due to instability in human plasma at low concentrations. 8-Cl-adenosine was determined using isocratic elution, the nucleotides were determined using gradient elution (see Experimental).

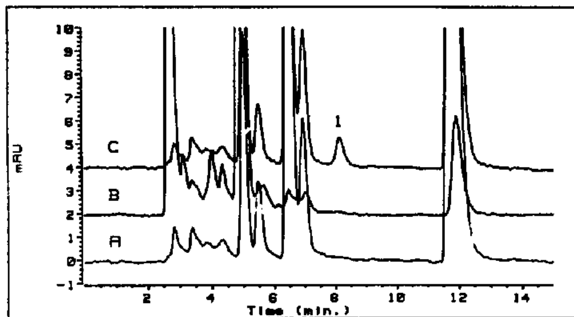

Fig. 2. Reversed-phase, isocratic elution HPLC of 8-Cl-adenosine. HPLC and SPE are described in Experimental. Chromatogram A is a blank plasma extract. Chromatogram B is a plasma extract from a patient treated with 25 μg/kg/h 8-Cl-cAMP as a continuous infusion. The blood sample was collected 4 h into the infusion but no peak due to 8-Cl-adenosine was present. Chromatogram C is a plasma extract spiked with 20 ng of 8-Cl-adenosine (peak 1).

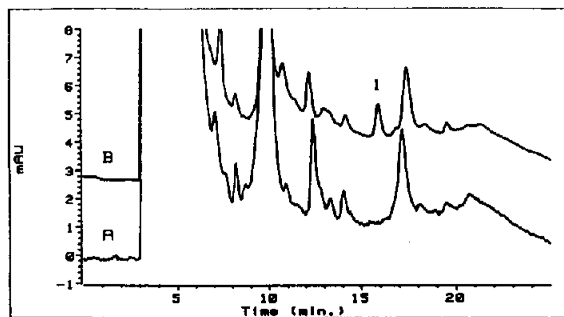

Fig. 3. Analysis of plasma samples from a patient treated with 8-Cl-cAMP as a continuous infusion at a dose of 25 μg/kg/h. Chromatogram A is a pre-dose specimen. Chromatogram B is a plasma specimen taken 4 h into the infusion. Peak 1 is 8-Cl-cAMP at a concentration of 47 ng/ml.

lesser extent the 5′-mononucleotide metabolites of 8-Cl-cAMP) were shown in preliminary studies to be highly unstable in human plasma ruling out their direct determination. This effect was concentration dependent, with greater instability being observed at lower concentrations. High percent recoveries were achieved from plasma spiked with the higher concentration of nucleotide standards (10 μg/ml), with the 5′-mononucleotides generally recording better extraction efficiencies than the cyclic nucleotides (Table 2). At the lower and biologically more relevant (see Fig. 4) concentration studied (100 ng/ml), the mononucleotide components were not detected due to their instability. However, no such problem occurred with the cyclic nucleotides, although there was a tendency towards a greater between-day C.V. in recovery. Previous studies have also reported that 8-Cl-cAMP is not degraded significantly by human plasma [8]. Almost quantitative recovery of 8-Cl-adenosine was achieved at the higher concentration (10 μg/ml) studied but this dropped to 67.8% ± 4.0 at 100 ng/ml. This latter result may be due to the fact that reversed-phase SPE of nucleosides does not always release material bound to plasma proteins [9], and this effect may become limiting at lower concentrations. Both SPE techniques produced chromatograms which were virtually free from co-eluting, endogenous, interfering peaks, even when run at high detector sensitivity (0.01 AU, full scale deflection, see Fig. 1 trace A and B for nucleotides and Fig. 2 A and C for 8-Cl-adenosine).

3.3. Analysis of patient samples

Chromatograms of plasma samples taken from a patient receiving 8-Cl-cAMP as a continuous infusion at a dose level of 25 μg/kg/h are shown in Fig. 3. While the pre-dose plasma sample

Table 2
Solid-phase extraction of 8-Cl-cAMP, its nucleotide metabolites and 8-Cl-adenosine from plasma

Concentration	IMP	8-Cl-IMP	AMP	8-Cl-AMP	cAMP	8-Cl-cAMP	8-Br-cAMP	8-Cl-adenosine
10 μg/ml	75.8 ± 2.7[a]	70.1 ± 10.3	77.6 ± 10.5	79.6 ± 8.4	68.6 ± 8.9	64.6 ± 18.6	61.1 ± 3.8	94.2 ± 4.2
0.1 μg/ml	NE	NE	NE	NE	69.8 ± 14.9	74.2 ± 19.4	58.6 ± 7.6	67.8 ± 4.0

[a] Mean percent recovery ± S.D. from 10–20 replicates run over a three week period.
NE, not evaluable due to instability in human plasma at low concentrations. 8-Cl-adenosine was extracted by reversed-phase (C_8) SPE and the nucleotides were extracted by strong anion-exchange SPE.

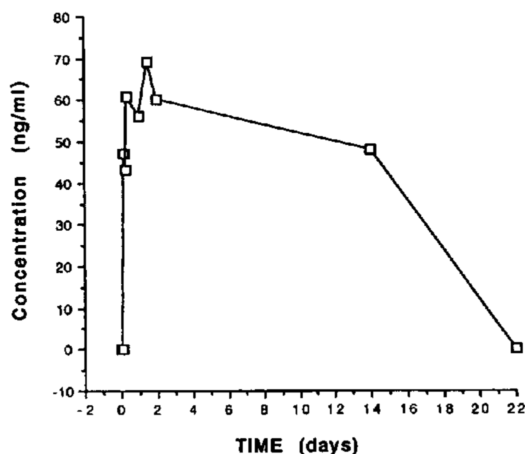

Fig. 4. Concentration–time profile of 8-Cl-cAMP in a patient treated with 8-Cl-cAMP as a continuous infusion at a dose of 25 μg/kg/h.

shows the presence of a number of interferences (Fig. 3A), the 8-Cl-cAMP peak is clearly discernable (47 ng/ml). Scanning the UV-spectrum of this peak with a diode-array detector confirmed that it had an absorption maximum at 261 nm (and correct spectral shift over cAMP), identical to a standard of 8-Cl-cAMP (see Table 1). Due to the close proximity of an interference to the 8-Cl-cAMP peak, 8-Br-cAMP could not be utilised as an internal standard in this sample and in some limited cases the external standard method of quantitation had to be applied. However, in the majority of clinical specimens analyzed, 8-Br-cAMP served as an ideal internal standard. At the same time point (4 h) as above and in the same patient, no 8-Cl-adenosine was detected (see Fig. 2B). A full pharmacokinetic profile of 8-Cl-cAMP concentrations obtained using the methodology described in this work is shown in Fig. 4. In this particular patient, at all the time points studied, no 8-Cl-adenosine was detected.

4. Discussion

The aim of this present study has been to develop sensitive analytical methodologies which would be capable of determining 8-Cl-cAMP and 8-Cl-adenosine in patient plasma samples after drug administration at a low dose continuous infusion. To achieve this aim emphasis was placed on sample preparation. Previous sample preparation procedures for 8-Cl-cAMP have all been based on extraction of tissue culture media or cancer cell pellets with 5–10% trichloroacetic acid [3,8,10,11], or 0.4 *M* perchloric acid [12], followed by either extraction of acid into ether or neutralisation of acid with trioctylamine/Freon or potassium bicarbonate. These procedures normally involved a number of dilution steps and were only suitable for drug determination in the high μ*M* range (>10 μ*M*, approximately 5 μg/ml), which is clearly well above the levels present in patients. In addition, these methods are time consuming and they do not lend themselves to rapid analysis of batches of samples generated in large scale pharmacokinetic studies. In the present study SPE has been chosen for sample preparation since this technique can involve concentration rather than dilution of samples and it can be automated. The results of this work show that the new techniques described are sufficiently sensitive to detect 8-Cl-cAMP in patient plasma after low dose drug administration and that in a single patient it appears that 8-Cl-adenosine is not formed to any significant extent. Confirmation of this finding will require data from several patients, and at present these studies are ongoing. Application of this new methodology will aid in the development of 8-Cl-cAMP as a potential new form of anticancer treatment.

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