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Short communication

Liquid chromatography method for the analysis of adenosine compounds

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Abstract

A newly available chromatography column packing material that employs hybrid particle technology was used to improve the analysis of adenosine compounds. Using a TBAS buffer/acetonitrile gradient this material permits separation of etheno-adenosine compounds in less than 4 min with excellent resolution and sensitivity (50 fmol). Variability of compound quantification is small (coefficients of variation 0.23±0.14% for 50 pmol and 1.70±0.53% for 0.5 pmol). The new method is well suited for the analysis of adenosine compounds in small biological samples and permits a high sample throughput in autosampler setups with high precision and reproducibility.

Keywords: Adenosine compounds

1. Introduction

The analysis of adenosine compounds, e.g. adenosine and adenine nucleotides, in biological samples is of great practical importance [1–6]. Traditional HPLC methods use either UV-detection of native adenosine compounds or fluorescence detection following derivatization to etheno-compounds. Such etheno-derivatives in conjunction with fluorescence detection offer a substantial increase in sensitivity [7]. However, a limitation of current HPLC techniques is still the time required for the individual HPLC run, which is in the order of 15–60 min. The present report is based on the use of a newly available hybrid particle technology, which permits to shorten the run time to less than 4 min with excellent peak resolution and compound sensitivity.

2. Experimental

The HPLC device used was a Waters Alliance 2690 system, consisting of pumps, eluent degasser, mixing chamber, and injection module. Samples were injected onto an XTerra MS C_{18} column (4.6× 50 mm, I.D. 3 mm, 5 μm particle size, 125 Å pore size; Waters Corp.), which makes use of porous hybrid particles surface-bonded with C_{18} material

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and was equipped with a precolumn (XTerra MS C_{18}, 5 μm). Eluent flow-rate was 1.5 ml/min throughout the analysis if not stated otherwise, the ambient temperature was around 20 °C and not specifically controlled. The eluents used were a tetrabutylammoniumhydrogensulfate (TBAS) buffer (5.7 m*M* TBAS, 30.5 m*M* KH_2PO_4 adjusted to pH 5.8 with 2 N KOH) and an acetonitrile buffer (acetonitrile:TBAS buffer, 2:1). Starting condition was 90% TBAS buffer, which was linearly reduced to 60% within 1 min after sample injection. This condition was maintained for 1.4 min. From run time 2.4 to 2.5 min the TBAS buffer fraction was increased from 60 to 90%. This condition (90% TBAS) was maintained for 1.5 min, when the system was ready for injection of the next sample. Thus, the individual run time was 4 min plus the time required for sample injection. Injection volumes possible in the system were 5–100 μl, typically however, a sample volume of 5 μl was used. Continuous degassing of the buffers was achieved by the degassing module built into the HPLC system. The fluorescence detector employed was a Merck-Hitachi F 1050 fluorescence spectrophotometer (flow cell capacity 12 μl) set to an excitation wavelength of 280 nm and an emission wavelength of 410 nm [7]. Using a sensitivity setting of 1 and a time constant of 0.3 the detector output signal was recorded via a SATIN-box (Waters Corp.) on a PC using Millenium 3.20 Software (Waters Corp.). Retention times as well as quantification of compounds were assessed using external standards of known concentrations (ϵAdo, ϵAMP, ϵADP, ϵATP).

Native adenosine compounds were derivatized following the procedure as described recently [8]. In brief, 150 μl of sample or standard were incubated with 80 μl chloracetaldehyde, 770 μl citrate phosphate buffer (62 and 76 m*M*, respectively; pH 4.0), and 2.0 ml Krebs–Henseleit buffer (pH 7.4) at 80 °C for 40 min. Derivatized samples were immediately cooled to 4 °C or frozen until analysis. To test the applicability of the HPLC method to biological sample analysis, eye lenses from guinea pig and sheep (25–260 mg dry mass) were used. Samples were homogenized in 5 ml 1 N $HClO_4$. The 45 000 *g* supernatant was neutralized with approximately 6.75 ml K_3PO_4 (1 *M*). Following another centrifugation with 45 000 *g* (4 °C) the supernatant was lyophilized and redissolved in 0.65–1.0 ml aqua bidest, of which 150 μl was used for the above described derivatization procedure. A 5-μl aliquot was used for HPLC analysis.

Data reported in this study are mean values±S.D.

3. Results and discussion

In Fig. 1a a typical chromatogram is shown for a standard sample containing ϵAdo, ϵAMP, ϵADP, and ϵATP in amounts of 25 pmol per 5 μl. As evident from the overlay chromatogram in Fig. 1a, the repeatability of the retention times and the quantification of the compounds under analysis were excellent. Average retention times for the compounds were 1.09 min for ϵAdo, 1.60 min for ϵAMP, 2.10 min for ϵADP, and 2.55 min for ϵATP. The coefficient of variation of the retention times was 0.76±0.18%. Adjusting flow-rate between 0.5 and 3.0 ml/min resulted in retention times for ϵ-ATP ranging from 6.5 to 1.7 min and had the following effects on compound sensitivity and peak resolution. Peak height was optimal at a flow-rate of 0.5 ml/min and 28–49% lower at a flow-rate of 3.0 ml/min (Table 1). At a flow-rate setting of 1.5 ml the peak height was 73–93% of that obtained at a flow-rate of 0.5 ml/min. Peak resolution tended to be highest at a flow-rate of 3.0 ml/min and was slightly diminished at 1.5 ml/min (Table 2). However, it should be noted that peak resolution of different compounds was always greater than 1. Thus, the choice of a flow-rate of 1.5 ml/min was a trade-off between optimal peak resolution, sensitivity and eluent consumption.

The method gave linear relationships between the signal intensity recorded and the amount of compound analyzed in the range of 1–50 pmol (Table 3). The r^2-values for six independent determinations were between 0.976 (ϵATP) and 1.000 (ϵAMP). There was no significant difference of the correlation coefficients when evaluating peak area or peak height as measures of signal intensity. The coefficient of variation of the HPLC quantification of known standards was 0.23±0.14% for an amount of 50 pmol and 1.70±0.53% for 0.5 pmol. In comparison, the coefficient of variation of the sample derivatization was 2.26±0.26% for compound

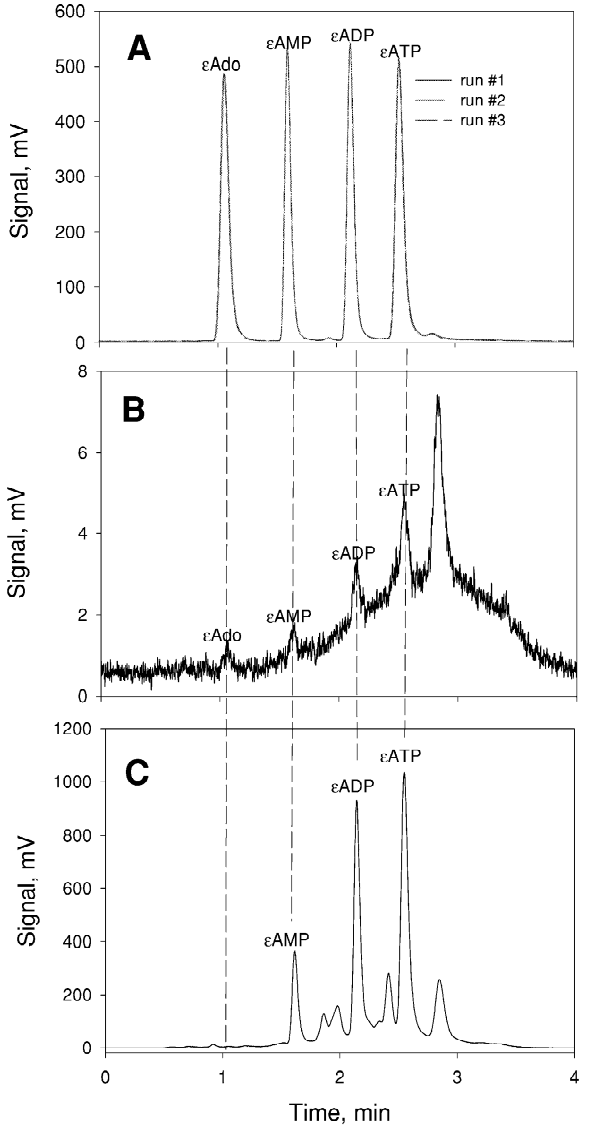

Fig. 1. Typical chromatograms of standards and biological sample. In (A) the repeatability of the method is shown for three injections of a standard of 25 pmol per compound. In (B) amounts of 50 fmol per compound are injected emphasizing the sensitivity of the method. (C) displays a chromatogram of a biological sample from sheep eye lens. ϵAdo: 1,*N*(6)-etheno-adenosine, ϵAMP: 1,*N*(6)-etheno-AMP, ϵADP: 1,*N*(6)-etheno-ADP, ϵATP: 1,*N*(6)-etheno-ATP.

amounts of 50 pmol, which includes the coefficient of variation of the HPLC procedure. The reproducibility of the technique proved to be reliable during daily routine analysis. Up to 260 samples of cell culture supernatant per day were analyzed without any significant loss of precision or resolution. As previous techniques have yielded HPLC run times in the range of 15–60 min [2,8,9,12,13], the newly available columns permit a significant reduction of the run time equivalent to a more than 6-fold increase of sample throughput.

The sensitivity of the method is excellent due to fluorescence detection of the derivatized material. As shown in Fig. 1b, a 10-μl aliquot of a standard with a concentration of 5 n*M*, equivalent to an amount of 50 fmol/10 μl per compound still resulted in an acceptable signal-to-noise ratio. In comparison, the sensitivity limit of traditional UV-detection is in the range of 10 pmol per sample [3,10,11]. Furthermore, the derivatization of the adenosine compounds permits a compound assessment in biological samples without disturbing co-eluting peaks of other compounds (Fig. 1c). Aliquots of an acid extract of total sheep lens clearly revealed the peaks of ϵAMP, ϵADP, and ϵATP. Similar results were obtained in guinea pig lens (Table 4). The levels of ethenonucleotides agreed well with those of adenine

Table 1
Peak height in response to variation of eluent flow-rate

Flow-rate (ml/min)	ϵAdo	ϵAMP	ϵADP	ϵATP
0.5	28.7	30.0	27.8	24.9
1.0	23.0	27.8	26.9	24.5
1.5	21.4	22.0	24.2	23.4
2.0	19.4	20.8	22.6	22.7
2.5	18.4	17.8	19.3	20.2
3.0	16.0	15.1	16.8	18.0

Data are in mV per 1 pmol of compound injected.

Table 2
Peak resolution in response to variation of eluent flow-rate

Flow-rate (ml/min)	ϵAdo ϵAMP	ϵAMP ϵADP	ϵADP ϵATP
0.5	1.11	1.38	1.16
1.0	1.55	1.45	1.17
1.5	1.64	1.71	1.23
2.0	1.72	1.81	1.28
2.5	1.65	2.00	1.46
3.0	1.88	2.31	1.32

Resolution of two adjacent peaks is given as the ratio of the sum of the peak half widths (PHW) and the difference of retention times (Δ*RT*) of the peaks (RES=Δ*RT*/(PHW1+PHW2).

Table 3
Correlation coefficients of calibration curves

Compound	r^2-value	Regression	Slope	Intercept
Height				
ϵAdo	0.998	$y=21\,804x-1759$	$P<0.0001$	$P=0.129$
ϵAMP	1.000	$y=21\,934x+887$	$P<0.0001$	$P=0.806$
ϵADP	0.992	$y=19\,220x+21\,333$	$P<0.0001$	$P=0.325$
ϵATP	0.976	$y=15\,801x+11\,759$	$P<0.0001$	$P=0.281$
Area				
ϵAdo	0.999	$y=101\,406x-40\,882$	$P<0.0001$	$P=0.052$
ϵAMP	0.999	$y=83\,972x-22\,104$	$P<0.0001$	$P=0.079$
ϵADP	0.994	$y=81\,034x+62\,043$	$P<0.0001$	$P=0.436$
ϵATP	0.978	$y=78\,102x+146\,307$	$P<0.0001$	$P=0.332$

Each regression and correlation coefficient is calculated from six measurements using amounts of 1, 5, 10, 20, 40, and 50 pmol per compound. Injection volumes were 10–100 μl.

nucleotides reported earlier for human eye lens [2]. Recoveries of adenine nucleotides and ϵ-adenine nucleotides were tested by adding the individual compounds to the acid extract that subsequently underwent the derivatization procedure. The recoveries were 104±13% for adenosine nucleotides and 120±21% for ϵ-adenosine nucleotides. The respective recoveries in standard samples were 105±12% and 95±24%.

It may be of interest to note that in the chromatogram shown in Fig. 1c, additional unknown peaks were visible, well separated from the derivatized adenosine compounds. It has been documented before that biological samples may contain a complex pattern of nucleotides [2]. In an attempt to evaluate which additional compounds can be analyzed by the method described, other adenine, guanosine and cytidine compounds were derivatized with the identical procedure and then chromatographed. Data are summarized in Table 5.

S-Adenosylhomocysteine and deoxy-ATP revealed single peaks. Also, GTP could be derivatized, however, there was obviously considerable breakdown of the triphosphate to guanosine. Thus, in the case of GTP etheno-derivatives were detectable at an early retention time similar to that of ϵAdo and at a late retention time close to that of ϵATP. It should be noted, however, that these latter peaks did not co-elute with those of the etheno derivatives of the adenosine compounds. CTP yielded detectable peaks only at a higher amount (2 nmol).

As evident from Table 5, ϵATP co-eluted with ϵdATP. As tissue samples may contain both fractions of nucleotides in relevant quantities it seems necessary to assess a possible interference of the com-

Table 5
Retention times of derivatized compounds

Compound	Retention time (min)
ϵAdoHcy	0.88
ϵAdo	1.09
ϵAMP	1.60
ϵADP	2.10
ϵGTP	2.30
ϵCTP	2.50
ϵdATP	2.55
ϵATP	2.55

ϵAdoHcy, etheno-*S*-adenosyl-homocysteine; ϵGTP, etheno-GTP; ϵCTP, etheno-CTP; ϵdATP, etheno-deoxy-ATP.
For other abbreviations, see legend to Fig. 1.

Table 4
Etheno-derivatized adenine nucleotides in acid extracts from guinea pig and sheep lens

Sample	ϵATP (μmol/g)	ϵADP (μmol/g)	ϵAMP (μmol/g)
Guinea pig lens	0.981	0.720	0.188
Sheep lens	0.995	0.879	0.357

Data are mean values in μmol per g dry mass from two lenses.

pounds if the method is extended to unknown tissue extracts. This has been achieved in the past by combining the chromatography method with a mass spectrometry detection technique [12,13]. Unfortunately, previous HPLC-methods have largely (20–60 min) accounted for the total analysis time. The present method reduces the necessary analysis time considerably and thereby makes the combined use of HPLC and mass spectrometry more efficient. In conclusion, we have evaluated a new rapid HPLC method for the analysis of adenosine compounds in biological samples which permits a high sample throughput in autosampler setups with high precision and repeatability.

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