ELSEVIER

Journal of Chromatography B, 707 (1998) 33–41

JOURNAL OF CHROMATOGRAPHY B

Simultaneous measurement of adenosine and hypoxanthine in human umbilical cord plasma using reversed-phase high-performance liquid chromatography with photodiode-array detection and on-line validation of peak purity

M. Helen Maguire*, István Szabó, István E. Valkó, Brent E. Finley, Timothy L. Bennett

Department of Pharmacology, Toxicology and Therapeutics and Ralph L. Smith Research Center and Department of Gynecology and Obstetrics, University of Kansas Medical Center, Kansas City, KS 66160-7417, USA

Received 3 June 1997; received in revised form 10 November 1997; accepted 17 November 1997

Abstract

A new, robust and sensitive reversed-phase high-performance liquid chromatographic method was developed for concomitant measurement of plasma concentrations of the ATP catabolites adenosine and hypoxanthine in human umbilical cord blood. Deproteinized cord plasma was chromatographed on Hypersil C_{18} columns, using UV photodiode-array detection, spectral analysis of peaks and on-line confirmation of peak purity. Elution with a gradient of acetonitrile–tetrahydrofuran in ammonium dihydrogen phosphate buffer pH 4.7, yielded sharp, well-resolved peaks of adenosine and hypoxanthine within 16 min. Peak areas were quantified from external calibration curves and converted to plasma concentrations via cord blood hematocrits. In seven deliveries, gestational ages 32–40 weeks, adenosine (range, 0.1–2.1 μM) was less than hypoxanthine (range, 1.6–18.5 μM) in the same cord plasma sample. Arteriovenous levels of each purine were similar, except in an abruptio placenta delivery. © 1998 Elsevier Science B.V.

Keywords: Adenosine; Hypoxanthine

1. Introduction

Breakdown of the energy-rich nucleotide ATP caused by tissue oxygen deficit yields adenosine and hypoxanthine as major products. In vivo, e.g., in brain, heart and kidney [1–3], regional ischemia results in localized release of adenosine into venous blood and extracellular fluids, increasing normally low extracellular concentrations of adenosine. Extracellular levels of the adenosine metabolite hypoxanthine also increase, because further metabolism of hypoxanthine, either by oxidation to xanthine and uric acid or by salvage to IMP, is inhibited in hypoxia [1–4]. Conversely, elevated extracellular levels of adenosine and hypoxanthine may signal the presence of localized tissue ischemia and hypoxia. Hypoxanthine has been proposed as a sensitive indicator of hypoxia that may be useful to predict hypoxic sequelae in vivo [4]. For these reasons there is interest in measuring plasma adenosine and hypoxanthine concentrations in human umbilical cord

*Corresponding author. Address for correspondence: Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160-7417, USA.

PII S0378-4347(97)00581-1

blood obtained at or prior to delivery. Elevated levels of these two purines in cord plasma may signify in utero hypoxia or fetal asphyxia during or prior to delivery, and indicate the need for special clinical care of the fetus or neonate.

Measurement of both adenosine and hypoxanthine concentrations in the same umbilical cord plasma sample has not been reported. Our objective was to develop a robust method for simultaneous measurement of adenosine and hypoxanthine in human umbilical cord plasma, using reversed-phase HPLC. Earlier studies showed that isocratic elution is not feasible for simultaneous analysis of adenosine and hypoxanthine in one chromatographic run because of the difference in hydrophilic and hydrophobic properties of the nucleoside and the purine base [5,6]. We report development of a robust gradient method for reversed-phase HPLC analysis of adenosine and hypoxanthine concentrations in deproteinized preparations of umbilical cord plasma using UV photodiode-array absorbance detection and on-line spectral analysis to validate analyte peak purity. Changes in analyte concentrations during plasma isolation were precluded by drawing umbilical cord blood into a stop solution. Umbilical cord blood hematocrits allowed calculation of plasma adenosine and hypoxanthine concentrations. Adenosine and hypoxanthine concentrations in umbilical cord arterial and venous plasma from seven patients delivered vaginally and by cesarean section are reported.

2. Experimental

2.1. Materials

Nucleosides and nucleobases, except xanthosine and xanthine, were supplied by Pharmacia P.L. Biochemicals (Piscataway, NJ, USA). Xanthosine, xanthine, allopurinol, uric acid, DL tryptophan and 100% trichloroacetic acid were obtained from Sigma Chemical Company (St. Louis, MO, USA). Dipyridamole was supplied by Geigy Pharmaceuticals (Ardsley, NY, USA). EHNA (*erythro*-9-2-hydroxy-3- nonyladenine hydrochloride) was generously provided by Burroughs Wellcome (now Glaxo–Wellcome) (Research Triangle Park, NC, USA). Reagent-grade 1,1,2-trichloro-1,2,2-trifluoroethane (Freon-113), HPLC-grade acetonitrile, HPLC-grade tetrahydrofuran and HPLC-grade 85% phosphoric acid were obtained from Fisher Scientific (Pittsburgh, PA, USA). Ammonium dihydrogen phosphate was Baker-analyzed HPLC-grade from J.T. Baker (Phillipsburgh, NJ, USA). Tri-*n*-octylamine was obtained from Aldrich (Milwaukee, WI, USA). Nylon Acrodisc 0.45 μm disposable membrane filters were supplied by Gelman Sciences (Ann Arbor, MI, USA). Distilled water was passed through an IWT charcoal cartridge and redistilled in an all glass system. All other chemicals were the best grade commercially available.

2.2. Instrumentation

HPLC was performed on a Varian 5560 chromatograph with column thermostat, 8085 autosampler, Rheodyne 7126 valve injector and Varian PolyChrome 9065 photodiode-array detector. A Spectra-Physics 4270 dual-channel data integrator with memory module was used to record analog chromatographic profiles and peak retention times (t_R), and to integrate peak areas and store chromatograms for reintegration. The PolyChrome 9065 detector was interfaced with a 486 IBM-compatible computer which acquired and processed chromatographic data via the Varian Star program, and analyzed peak spectral data via Varian PolyView software. Data collection frequency was 11 Hz with a bunch size of 4.

2.3. Evaluation of peak purity

Spectral wavelengths of chromatographic peaks were monitored continuously from 220 to 311 nm. The purity of peaks in chromatograms of deproteinized preparations of cord plasma was evaluated on-line from the spectral data by analysis of peak spectral identity with that of a reference standard, and by measurement of peak purity parameters. Spectral searches were carried out during the chromatographic run to determine the degree of identity of a peak area spectrum with that of a reference standard stored in a library in the computer, using multicomponent analysis; identity was expressed in the form of similarity and dissimilarity indices, with identical spectra having similarity and dissimilarity

indices of 1 and 0, respectively [7]. The purity parameter of a chromatographic peak represents the average wavelength of the peak spectrum within a specified wavelength range [8–10]. In pure peaks, upslope, apex and downslope purity parameters are identical. Apex purity parameters were compared with those of standards under identical chromatographic conditions. Agreement within a range of ±1 nm was considered evidence of a pure peak. Purity parameters and similarity–dissimilarity indices were printed in postrun reports.

2.4. Chromatographic conditions

Columns used were 150×4.6 mm I.D. and 250×4.6 mm I.D., packed with Shandon C_{18} Hypersil, particle size 5 or 3 μm, obtained from Keystone Scientific (State College, PA, USA). Brownlee guard cartridges, 15×3.2 mm I.D., packed with 7 μm, wide-pore spherical C_{18} silica were used routinely. Injection was performed manually or automatically using a 20 μl loop, unless otherwise noted. The low strength eluent of the mobile phase was ammonium dihydrogen phosphate buffer, and the pH was adjusted with potassium hydroxide or phosphoric acid. Solutions were filtered through a 0.45 μm Millipore membrane. The high strength eluent was either acetonitrile or acetonitrile containing tetrahydrofuran. Details of the mobile phase composition are given in Section 3.1. Mobile phases were degassed by sonication. Columns were thermostatted at 40°C. Flow-rate was 1.0 ml/min.

2.5. Preparation of deproteinized plasma

The umbilical cord was doubly clamped immediately after delivery and divided between the clamps. The divided cord was doubly clamped toward the placenta; using a 22 gauge needle, 2 ml of arterial and venous blood were drawn from the isolated vessel segments into heparinized syringes, each containing 1 ml of stop solution (see below), to give a total volume of 3 ml. Alternatively, 0.66 ml of blood was drawn into a 1 ml heparinized syringe containing 0.33 ml stop solution. Syringes were immediately inverted to mix blood and stopping solution, and samples were centrifuged at 3300 *g* for 10 min. Supernatants showing indication of lysis were discarded. Supernatants were transferred to glass centrifuge tubes, and vortex-mixed with a one fourth volume of 24% (v/v) trichloroacetic acid (TCA) for 10 s. Denatured proteins were centrifuged for 10 min at 1000 *g*. Supernatants were vortex-mixed for 30 s with 2 volumes of 0.5 *M* tri-*n*-octylamine in Freon-113, to extract TCA, aspirated and filtered through 0.45 μm Acrodisc membrane filters to yield the TCA-treated plasma samples, which were stored at −20°C until analysis by HPLC.

Blood was collected for determination of hematocrits.

2.6. Stop solution

Stop solution contained 75 μ*M* dipyridamole, 15 μ*M* EHNA and 3 m*M* disodium EDTA in isotonic saline, pH 6.0. When adenosine and hypoxanthine were to be quantified, 10 μ*M* allopurinol was present as an internal standard to correct for losses that might occur during sample handling.

3. Results

3.1. Development of chromatographic method

Chromatography of TCA-treated cord arterial plasma on a 150×4.5 mm I.D. column of C_{18} Hypersil, particle size 3 μm, using a concave gradient of acetonitrile in 0.0075 *M* ammonium dihydrogen phosphate pH 6.0, and detection wavelength 254 nm cf. [5,6], yielded a base-line resolved peak of hypoxanthine, t_R 4.58 min, but the adenosine peak, t_R 11.64 min, coeluted with a major contaminant characterized by strong absorption at lower wavelengths and was not resolved from a small peak on the trailing edge. Use of a longer C_{18} Hypersil column, particle size 5 μm, yielded a pure peak of hypoxanthine, t_R 7.17 min, and a well-resolved but impure peak of adenosine, t_R 13.07 min, that was followed by a major peak, identified by t_R and spectra as tryptophan (Fig. 1a). As shown in Fig. 1b, increasing the molarity and decreasing the pH of the low strength eluent shifted the tryptophan peak, but the adenosine peak was not well-resolved; the hypoxanthine peak, although well-resolved, was not pure. Subsequent studies addressed the need to achieve

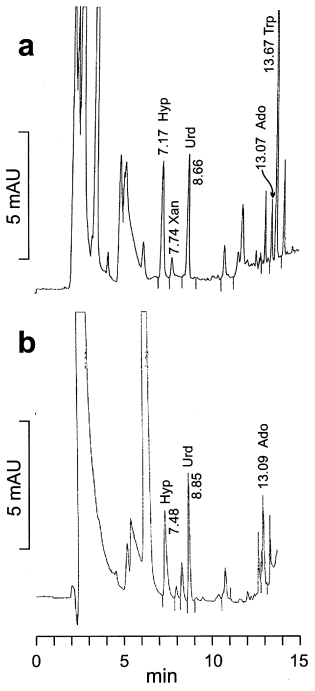

Fig. 1. Chromatograms of TCA-treated umbilical cord arterial plasma. Injection volume, 40 μl. Detection wavelength, 254 nm. Identified peaks are labelled. (a) Column, 250×4.5 mm I.D. C_{18} Hypersil, 5 μm particle size; chromatographic method, concave gradient of acetonitrile in 0.0075 *M* ammonium dihydrogen phosphate pH 6.0. (b) Column and sample, same as (a); chromatographic method, concave gradient of acetonitrile in 0.05 *M* ammonium dihydrogen phosphate pH 3.3. Hypoxanthine (Hyp) and xanthine (Xan) peaks in (a), and the uridine (Urd) peak in (b) were pure as indicated by purity parameters.

robust resolution of adenosine and tryptophan and obtain pure peaks of adenosine and hypoxanthine. The original low strength eluent, 0.0075 *M* ammonium dihydrogen phosphate pH 6.0, was used, but the shape of the acetonitrile gradient was modified and the maximum concentration of acetonitrile reached in the mobile phase was reduced to increase the elution time of tryptophan. To sharpen peaks, inclusion of tetrahydrofuran, 0.3 to 2%, in the organic modifier was evaluated; 1% tetrahydrofuran proved optimal. A linear gradient of water–acetonitrile–tetrahydrofuran (49:50:1, v/v/v) in 0.0075 *M* ammonium dihydrogen phosphate pH 6.0 gave improved resolution of hypoxanthine and adenosine, a flat baseline, and was robust. Detection at 263 nm increased the size of the adenosine peak and reduced the absorption of the sample matrix. Study of the effects of reducing the pH and increasing the ionic strength of the ammonium dihydrogen phosphate buffer showed that pH 4.7 and 0.03 *M*, respectively, were optimal. Resolution of hypoxanthine was enhanced by detection at 249 nm, and change of C_{18} Hypersil particle size from 5 to 3 μm further improved resolution of analyte peaks from minor matrix peaks. Fig. 2a shows the baseline resolution

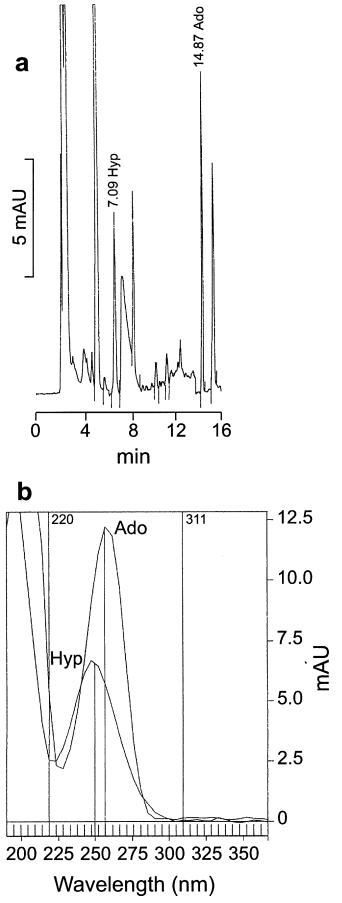

Fig. 2. (a) Chromatogram of TCA-treated fetal unbilical cord venous plasma. Injection volume 20 μl. Column, 250×4.5 mm I.D. C_{18} Hypersil, 3 μm particle size; chromatographic method, linear gradient of water–acetonitrile–tetrahydrofuran (49:50:1, v/v/v) in 0.03 *M* ammonium dihydrogen phosphate pH 4.7. Detection wavelength, 249 nm at 0–9.5 min and 263 nm at 9.5–18 min. (b) Spectra of the hypoxanthine (Hyp) and adenosine (Ado) peaks shown in (a). Upslope, apex and downslope purity parameters were: hypoxanthine peak, 249.99, 249.51 and 249.65 nm; adenosine peak, 256.16, 256.18 and 255.96 nm.

of adenosine and hypoxanthine achieved by these conditions in the chromatogram of a sample of TCA-treated fetal cord venous plasma. Spectra of the peaks are shown in Fig. 2b, and upslope, apex and downslope purity parameters for the peaks, given in the legend to Fig. 2b, confirmed the purity of the peaks. Purity parameters of standards are listed in Table 1.

3.2. Optimized chromatographic method

The optimized chromatographic method used 0.03 *M* ammonium dihydrogen phosphate pH 4.7 as the low strength eluent (A) and water–acetonitrile–tetrahydrofuran (49:50:1, v/v/v) as the organic modifier (B). The gradient program was 0–15 min, 0–18% B; 15–16 min, 18–0% B; reequilibration time at 0% B, 15 min. Detection wavelengths were: 249 nm at 0–9.0 min and 263 nm at 9.0–18 min. Detection limits at $S/N=3$, were adenosine 5.65 n*M*, i.e. 113 fmol/20 μl injection, and hypoxanthine 14.7 n*M*, i.e. 294 fmol/20 μl injection.

Typical chromatograms of TCA-treated umbilical cord venous and arterial plasma illustrate the baseline resolution of adenosine, hypoxanthine and allopurinol peaks achieved by the optimized method, cf. Fig. 3a and Fig. 4a. A chromatogram of standards of adenosine, hypoxanthine and allopurinol is shown in Fig. 3b, and t_R values and purity parameters of the standards are listed in Table 1. Similarity–dissimilarity indices and upslope, apex and downslope purity parameters of the analyte peaks in the chromatogram shown in Fig. 3a indicated that the analyte peaks were pure, cf. legend. Fig. 4b shows area spectra of the hypoxanthine and adenosine peaks in the chromatogram in Fig. 4a, overlaid with the spectra of standards; similarity–dissimilarity indices, cf. legend, indicated that the peaks were pure.

Table 1
Retention times and purity parameters of purine standards

Analyte	Retention time (mean±S.D., $n=5$) (min)	Purity parameter[a] (mean±S.D., $n=5$) (nm)
Hypoxanthine	6.97±0.03	248.95±0.17
Allopurinol	9.38±0.08	242.68±0.02
Adenosine	14.74±0.04	256.37±0.12

Standards were chromatographed under the optimized chromatographic conditions described in the text.
[a] Peak apex, 220–311 nm range.

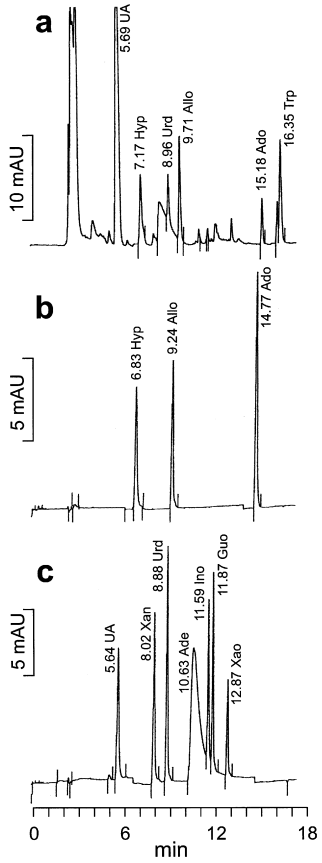

Fig. 3. Chromatograms obtained using the column and chromatographic method described in the legend to Fig. 2a. Injection volume 20 μl. Detection wavelength was 249 nm at 0–9.0 min, and 263 nm at 9.0–18 min. (a) Chromatogram of TCA-treated umbilical cord venous plasma. Upslope, apex and downslope purity parameters of the hypoxanthine peak and the adenosine peak were, respectively, 249.90, 249.39 and 249.61 nm, and 255.99, 256.38 and 256.41 nm. Allopurinol and uric acid peaks were spectrally pure. (b) Chromatogram of standards of hypoxanthine (Hyp) 103 pmol, allopurinol (Allo) 117 pmol and adenosine (Ado) 99 pmol. (c) Chromatogram of standards of uric acid (UA) 268 pmol, xanthine (Xan) 296 pmol, uridine (Urd) 328 pmol, adenine (Ade) 889 pmol, inosine (Ino) 168 pmol, guanosine (Guo) 159 pmol and xanthosine (Xao) 158 pmol.

A chromatogram of standards of other purines, and the pyrimidine uridine, is shown in Fig. 3c. Inosine, identified by t_R (11.56±0.03 min, $n=5$) and similarity–dissimilarity indices, was present in chromatograms of some plasma extracts, but generally

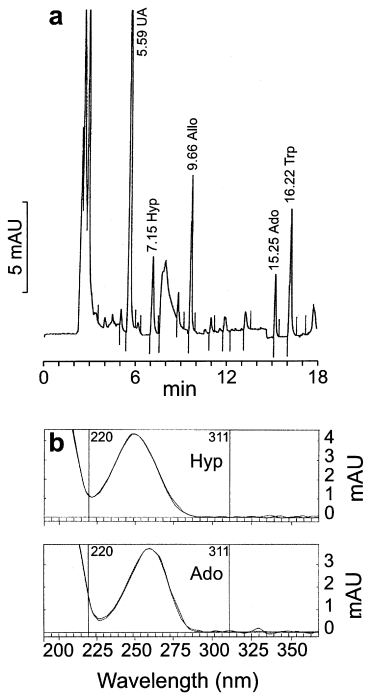

Fig. 4. (a) Chromatogram of TCA-treated umbilical cord arterial plasma. Chromatographic conditions are described in the legend to Fig. 3. (b) Upper frame, overlay of the area spectrum of the hypoxanthine (Hyp) peak and that of a standard; lower frame, similar data for the adenosine (Ado) peak. Similarity–dissimilarity indices for the peaks were, respectively, hypoxanthine, 0.99976–0.02173 and adenosine, 0.99944–0.03348.

the peak was too small for confirmation of peak purity. Xanthine (t_R=7.99±0.06 min, n=5), was not resolved in optimized chromatograms of TCA-treated plasmas, but was identified as a minor peak in chromatograms obtained using different conditions, cf. Fig. 1a. Guanosine and guanine were not found in TCA-treated cord plasma and the presence of xanthosine and adenine was not confirmed. Uric acid (t_R=5.57±0.04, n=5) was a prominent, well-resolved, pure peak in all chromatograms. Above t_R values are means±S.D..

3.3. Calibration curves

Calibration curves relating analyte concentration in TCA-treated plasma and peak area were linear over concentrations and at specific detection wavelengths, as follows (S.D.=standard deviation): hypoxanthine, 0.25–16.3 μ*M* at 249 nm; adenosine, 0.2–6.85 μ*M* at 263 nm; allopurinol, 1.25–11.7 μ*M* at 263 nm. Each curve contained 4–7 concentrations of analyte standards. Dependence of peak area (y) on concentration (x) was described by the following equations (n=number of calibration curves): $\text{hypoxanthine}_{249\ nm}$, $y=419x-407$, $\text{S.D.}_{slope}=9.5$, $\text{S.D.}_{intercept}=800$, $n=4$; $\text{allopurinol}_{263\ nm}$, $y=348x-1538$, $\text{S.D.}_{slope}=29$, $\text{S.D.}_{intercept}=1375$, $n=3$; $\text{adenosine}_{263\ nm}$, $y=631x+471$, $\text{S.D.}_{slope}=10.1$, $\text{S.D.}_{intercept}=1041$, $n=4$. Linear correlation coefficients were >0.999 for each individual calibration curve.

3.4. Quantitation

TCA-treated plasma preparations were chromatographed in duplicate or triplicate using automated or manual injection and 20 μl loops. Relative standard deviation (R.S.D.) values of mean values of triplicate injections were less than 10% and means of duplicate values differed from individual values by less than 5%. Peak areas were converted to analyte concentrations in TCA-treated plasma via calibration curves, and concentrations in TCA-treated plasma were corrected for dilution and hematocrit to yield cord plasma concentrations.

3.5. Analyte recoveries

Analyte recoveries were determined by spiking cord plasma-stop solution with known amounts of adenosine, hypoxanthine and allopurinol and quantifying the amounts recovered in TCA-treated plasma. Samples of plasma-stop solution were divided into two aliquots of 700 μl each. One aliquot was spiked with known amounts of adenosine, hypoxanthine and allopurinol, both spiked and unspiked aliquots were processed to yield TCA-treated plasma and analyte concentrations in both samples were determined. The concentrations of the adenosine, hypoxanthine and allopurinol spikes were quantified by HPLC. HPLC measurements were performed in triplicate.

Table 2 shows levels of adenosine and hypoxanthine in four different plasma-stop solution samples,

Table 2
Recovery of adenosine and hypoxanthine from umbilical cord plasma

Patient no./ plasma	Adenosine				Hypoxanthine			
	Concentration (μM)			Recovery (%)	Concentration (μM)			Recovery (%)
	Plasma-stop solution	Spike	Spiked plasma-stop solution		Plasma stop solution	Spike	Spiked plasma-stop solution	
1/venous	0.328	0.257	0.565	96.6	0.98	0.96	1.99	102.3
2/arterial	0.386	0.595	1.02	104.0	2.11	2.47	4.57	99.9
3/arterial	3.385	5.0	8.52	101.6	3.50	11.98	14.94	96.5
4/arterial	0.685	1.615	2.49	108.3	17.0	24.81	42.53	101.7

Umbilical cord plasma-stop solution from cord blood of four patients was aliquotted into two samples; one sample was spiked with known amounts of adenosine and hypoxanthine. Spiked and unspiked samples were processed to give TCA-treated plasma which was analyzed using the optimized chromatographic conditions as described in the text. Triplicate injections were performed; R.S.D. of means of triplicate values was $<10\%$.

amounts of adenosine and hypoxanthine with which the samples were spiked and concentrations of adenosine and hypoxanthine in the spiked samples. Recoveries of adenosine and hypoxanthine are expressed as percentage of added analytes. Mean overall recoveries$\pm$S.D. were adenosine, 102.6$\pm$4.9% and hypoxanthine, 100.1$\pm$2.6%.

The allopurinol spike and the percentage recoveries of allopurinol in samples from patients numbers 1, 2 and 3 in Table 2 were, respectively: 5.5 μM, 106.3%, 10.38 μM, 92.5% and 18.04 μM, 98.9%, giving an overall mean recovery$\pm$S.D. of 99.2$\pm$6.9%.

3.6. Umbilical cord plasma levels of adenosine and hypoxanthine

Levels of adenosine and hypoxanthine in umbilical cord arterial and venous plasma from seven patients, gestational ages 32 to 40 weeks, are shown in Table 3. The newborns were healthy and were not acidotic. No relationship is apparent between gestational age and levels of either arterial or venous plasma purines. In cord plasma from individual patients, hypoxanthine concentrations were higher than adenosine concentrations. However, there was no substantial arteriovenous difference in either purine, with the

Table 3
Concentrations of adenosine and hypoxanthine in human umbilical cord plasma

Patient no.	Gestational age (weeks)	Delivery type	Adenosine (μM)		Hypoxanthine (μM)	
			Artery	Vein	Artery	Vein
1	32	Cesarean section	0.74	0.82	6.85	6.45
2	34	Cesarean section	1.56	1.42	3.16	3.22
3[a]	35	Cesarean section	2.10	0.55	8.19	18.50
4	37	Cesarean section	0.34	0.29	2.54	2.40
5	37	Cesarean section	0.26	0.19	3.84	4.99
6	38	Vaginal	0.07	0.06	1.62	1.60
7[b]	40	Vaginal	1.69	1.06	14.1	12.9

[a] Abruptio placenta.
[b] Preeclampsia.

exception of patient number 3, where abruptio placenta occurred. Levels of adenosine and hypoxanthine in umbilical cord venous plasma obtained by percutaneous umbilical cord blood sampling at 20 weeks gestation from a pregnancy complicated by maternal diabetes and fetal ascites, were 2.8 and 4.4 μM, respectively (cf. Fig. 2).

4. Discussion

Extracellular levels of the purines adenosine and hypoxanthine reflect regional tissue ischemia, and increased plasma levels of these purines in human umbilical cord blood prior to or at delivery may indicate fetal hypoxia or asphyxia during delivery. Measurement of plasma adenosine and hypoxanthine concentrations in cord blood may be useful in diagnosing needs of the fetus or neonate for special clinical care. The objective of this study was to develop a facile and reliable method for measurement of plasma levels of adenosine and hypoxanthine in human umbilical cord blood, using reversed-phase HPLC as the analytical tool and deproteinized plasma as the analysis sample. To prevent changes in plasma levels of the purines during drawing of cord blood and isolation of plasma, blood was drawn into a stop solution in a volume ratio of 2:1 to facilitate rapid mixing, and the stop solution contained dipyridamole, an inhibitor of adenosine uptake into erythrocytes [11], EHNA, an inhibitor of adenosine deaminase [12], and EDTA to inhibit release of hypoxanthine by erythrocytes [13,14]. Furthermore, allopurinol was present as an internal standard to correct for any losses in sample handling. Treatment of the plasma-stop solution mix with TCA, a protein-denaturing agent, removed plasma proteins and yielded the analysis sample for measurement of the purines. Plasma concentrations of adenosine and hypoxanthine were calculated via cord blood hematocrits.

A reversed-phase HPLC method for simultaneous measurement of adenosine and hypoxanthine in the deproteinized plasma samples was developed and optimized. UV photodiode-array detection allowed continuous monitoring of spectral wavelengths of chromatographic peaks, and appropriate software provided numerical on-line assessment of chromatographic peak purity in the form of similarity–dissimilarity indices and purity parameters. Chromatograms of cord plasma extracts contained many major and minor peaks, and on-line measurement of peak purity was central to method development, assisting in peak identification, providing evidence for coeluting peaks and confirming peak purity.

In the optimized chromatographic method, TCA-treated plasma was chromatographed on C_{18} Hypersil columns using a linear gradient of the organic modifier, acetonitrile, in ammonium dihydrogen phosphate buffer. Tetrahydrofuran in the acetonitrile, and optimal pH and ionic strength of ammonium dihydrogen phosphate, served to sharpen analyte peaks and achieve baseline resolution from other peaks in the sample matrix. Elution of adenosine and hypoxanthine and the internal standard, allopurinol, was achieved within 16 min, with a column equilibration time of 15 min. Detection of hypoxanthine and adenosine at 249 and 263 nm, respectively, increased the size of the analyte peaks and the sensitivity of detection, which at S/N of 3, was <15 nM for hypoxanthine and <6 nM for adenosine. The optimized chromatographic method was robust, giving reproducible t_R values for the analytes and a stable, flat baseline. Purity of the analyte peaks was automatically validated on-line, as described above, to ensure that there was no interference from unknown peaks in the sample matrix, levels of which varied between patients, and no changes in resolution, and thus in peak purity with column aging. Quantification of the analytes was performed from external calibration curves. Recoveries of known amounts of adenosine and hypoxanthine from plasma-stop solution were 99–102% indicating the reproducibility of the protocol. This is the first report of concomitant measurement of adenosine and hypoxanthine in deproteinized human cord plasma in a single chromatographic run, and the first report of measurement of both purines in the same plasma sample.

In umbilical cord arterial and venous plasma of seven patients who delivered at 32 to 40 weeks gestation, either vaginally or by cesarean section, hypoxanthine levels were greater than adenosine levels in the same plasma sample. Although adenosine levels varied widely between different patients, from less than 0.1 to 2.1 μM, there was no substan-

tial arteriovenous difference in adenosine except in the case of abruptio placenta where arterial adenosine was almost four times the venous adenosine level. Nor were substantial arteriovenous differences in plasma hypoxanthine observed, except for the abruptio placenta delivery where the level of venous plasma hypoxanthine was more than twice that in arterial plasma. The highest levels of plasma adenosine and hypoxanthine were found in the abruptio placenta delivery. Abruptio placenta, which is characterized by separation of the placenta from the uterine wall is likely to result in fetal ischemia and hypoxia, and increased levels of adenosine and hypoxanthine in cord plasma may therefore be anticipated, and were observed. Furthermore, cord arterial and venous plasma levels of hypoxanthine were high in a delivery with a diagnosis of preeclampsia (cf. Table 3), a condition which may cause fetal hypoxia [15].

The chromatographic method was developed specifically for simultaneous determination of adenosine and hypoxanthine levels in human umbilical cord plasma, but may have more general application for concomitant measurement of these purines in plasma from other sites. In a preliminary study using the protocols reported here, concentrations of plasma adenosine and hypoxanthine in blood drawn from the cubital vein of an adult female were 0.08 and 0.235 μM, respectively (unpublished findings). The adenosine concentration is in agreement with the reported level of 92 ± 8 nM [12]; reliable plasma hypoxanthine levels for comparison do not appear to be reported.

In conclusion, we report for the first time a facile and precise chromatographic method for concomitant quantitation of plasma levels of adenosine and hypoxanthine in human arterial and venous umbilical cord blood. Reliable measurement of these two purines, which are putative indicators of fetal hypoxia, will provide another descriptor of fetal conditions as an adjunct to clinical diagnosis.

Acknowledgements

These studies were supported by National Institutes of Health Grants, HD 14888 and HD 02528.

References

[1] D.G.L. Wylen, T.S. Park, R.M. Berne, J. Cereb. Blood Flow Metab. 6 (1986) 522–528.
[2] A. Edlund, B. Berglund, D. Van Dorne, L. Kaiser, J. Nowak, C. Patrono, A. Sollevi, A. Wennmalm, Circulation 71 (1985) 1113–1120.
[3] W.L. Miller, R.A. Thomas, R.M. Berne, R. Rubio, Circ. Res. 43 (1978) 390–397.
[4] O.D. Saugstadt, Pediat. Res. 23 (1988) 143–150.
[5] M.H. Maguire, I. Szabo, P. Slegel, J. Liq. Chromatogr. 12 (1989) 2303–2320.
[6] M.H. Maguire, I. Szabo, P. Slegel, C.R. King, J. Chromatogr. 575 (1992) 243–253.
[7] J.-L. Excoffier, M. Joseph, J.J. Robinson, T.L. Sheehan, J. Chromatogr. 631 (1993) 15–21.
[8] T. Alfredson, T. Sheehan, T. Lenert, S. Aamodt, L. Correia, J. Chromatogr. 385 (1987) 213–223.
[9] J.J. Robinson, M.E. Adaskaveg, J.-L. Excoffier, Proc. Pittsburgh Conf. Exposition Anal. Chem. Appl. Spectrosc., 1989, Abstract 807.
[10] I. Szabo, M.H. Maguire, Anal. Biochem. 215 (1993) 253–260.
[11] J. Ontyd, J. Schrader, J. Chromatogr. 307 (1984) 404–409.
[12] G.H. Moser, J. Schrader, A. Duessen, Am. J. Physiol. 256 (1989) 799–806.
[13] R. Boulieu, C. Bory, P. Baltassat, C. Gonnet, Anal. Biochem. 129 (1983) 398–409.
[14] C. Lartigue-Mattei, J.L. Chabard, H. Bargnoux, J. Petit, J.A. Berger, J.M. Ristori, J.L. Bussiere, S. Rampon, Ann. Biol. Clin. 42 (1984) 355–361.
[15] C.M. Bilardo, K.H. Nicolaides, S. Campbell, Am. J. Obstet. Gynecol. 102 (1990) 115–120.