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MEASUREMENT OF ADENOSINE, INOSINE AND HYPOXANTHINE IN HUMAN TERM PLACENTA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An analytical method was developed for measuring adenosine, inosine and hypoxanthine in freshly delivered human term placentas. Representative freeze-clamped samples were taken from the sub-maternal surface of each placenta. Acid-soluble extracts of the samples were analyzed by reversed-phase high-performance liquid chromatography on columns packed with 10-μm porous octadecylsilica, using gradient elution with a linear increase in methanol concentration in ammonium phosphate buffer. Resolution of hypoxanthine from xanthine and adenosine from adenine, and quantitation of hypoxanthine and adenosine were achieved using 0.05 *M* ammonium dihydrogen phosphate, pH 6.5, as the low-strength eluent. Resolution of inosine from a prominent peak of β-NAD was optimized using 0.02 *M* ammonium dihydrogen phosphate, pH 5.6, as low-strength eluent. Recovery of standards was > 90%. Mean contents (± S.D.) of the analytes in placentas from seven normal deliveries were, adenosine 30.6 ± 11.5 nmol/g, inosine 68.0 ± 25.8 nmol/g and hypoxanthine 217 ± 127.5 nmol/g.

INTRODUCTION

The adenine nucleotide metabolite adenosine is vasoactive and has a number of putative physiological roles, particularly as a metabolically linked regulator

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of blood flow in certain tissues [1]. In human term placenta it was shown that levels of enzymes metabolizing adenine nucleotides were such as to allow accumulation of adenosine, especially under conditions of hypoxia [2, 3]. Aqueous heat-denatured homogenates of term placenta were shown to contain adenosine and its metabolites inosine and hypoxanthine, which were present in greater amounts than adenosine [2]. The presence of adenosine in human term placenta may be of significance to the regulation of placental function. In this study, improved analytical techniques have been used for measurement of adenosine, inosine and hypoxanthine in human term placenta and the levels of these metabolites in term placenta have been re-evaluated. Placental tissue was sampled by freeze-clamping, acid-soluble tissue extracts were prepared and a reversed-phase high-performance liquid chromatographic (HPLC) method was developed for analysis of the extracts.

EXPERIMENTAL

Materials

Nucleosides and nucleobases were obtained from Pharmacia P.-L. Biochemicals (Piscataway, NJ, U.S.A.) and β-NAD, xanthine oxidase, purine nucleoside phosphorylase, adenosine deaminase and NADase from Sigma (St. Louis, MO, U.S.A.). Fisher Scientific HPLC-grade methanol, 100% (w/v) trichloroacetic acid and reagent-grade 1,1,2-trichloro-1,2,2-trifluoroethane (Freon) were used. Baker-analyzed HPLC-grade ammonium dihydrogen phosphate was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.), and tri-*n*-octylamine (Alamine 336) from McKerson (Minneapolis, MI, U.S.A.) and from Aldrich (Milwaukee, WI, U.S.A.). Ammonium hydroxide was Mallinckrodt reagent grade. [8-^{14}C]Adenosine (55 mCi/mmol), [8-^{14}C]inosine (52 mCi/mmol) and [8-^{14}C]hypoxanthine (54 mCi/mmol) were purchased from Amersham (Arlington Heights, IL, U.S.A.). Affi-Gel-601 was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Instrumentation

A Varian 8500 chromatograph with solvent programmer, high-pressure septumless injector and Varian UV-50 variable-wavelength detector was used, together with either a Varian A-25 chart recorder or a Varian 4270 chromatography integrator. The sample flow cell temperature was maintained at 25°C. Samples were injected by a Hamilton syringe with a blunt 22 gauge needle. Analyses were performed on Varian MCH-10 columns, 300 × 4 mm I.D., factory-packed with 10-μm porous octadecylsilica. Manufacturer-specified theoretical plate numbers for the columns, based on elution of anthracene, ranged from 5265 to 7001. A 40 × 4 mm I.D. guard column, dry-packed with Vydac 40-μm pellicular reversed-phase packing, was used to protect the analytical column. When chromatographic profiles were recorded on the Varian A-25 chart recorder, retention times and peak heights were measured manually; calibration curves of peak height versus amounts of injected standards were fitted by regression analysis. The Model 4270 data integrator recorded chromatographic profiles, peak retention times and integrated peak areas, and performed linear regression analysis for calibration equations of peak area versus amounts of standards.

HPLC conditions

Two mobile phase systems which had a linear gradient in methanol were used. In one mobile phase, the low-strength eluent was 0.05 *M* ammonium dihydrogen phosphate, pH 6.5, and the high-strength eluent was methanol—water (60:43); in the second mobile phase, 0.02 *M* ammonium dihydrogen phosphate, pH 5.6, was the low-strength eluent and the high-strength eluent was methanol—water (70:33). The pH of ammonium dihydrogen phosphate solution was adjusted to the required value with ammonium hydroxide, and the solutions were filtered through a 0.45-μm Millipore membrane. Mobile phases were degassed by sonication. Distilled water was passed through IWT ion-exchange and charcoal cartridges and then redistilled in an all-glass system. Mobile phase was pumped at 1 ml/min. Injection volumes of samples and standards were 15—50 μl.

Peak identification

Peaks in HPLC profiles of placental extracts were identified by comparison of retention times (t_R) with those of standards, by the increase in peak height which occurred when a known standard was coinjected with the placental extract, by comparison of absorbance ratios (A_{250}/A_{260}) with those of standards, and by enzymatic peak shifts. Enzymes used for peak shifts were adenosine deaminase, purine nucleoside phosphorylase, xanthine oxidase and NADase; optimum pH and buffer composition were used for enzyme assays [4]. For determination of absorbance ratios, triplicate measurements were made at each wavelength. Absorbance ratios and enzymatic peak shifts were also used to establish peak purity.

Quantitation

Calibration curves related peak height or integrated peak area to the amount of adenosine, inosine or hypoxanthine injected over the range of 100—500 pmol. Relative standard deviation of mean peak heights and integrated peak areas from triplicate injections of the standards was $< 3\%$. Linear regression analysis of the dependence of peak height or area on concentration of the standards gave correlation coefficients > 0.998. Chromatograms of placental extracts were analyzed in triplicate. Measured amounts of adenosine, inosine and hypoxanthine were expressed as nmol/g of tissue.

Prediction of retention

Predicted retention times for inosine, hypoxanthine, xanthine and β-NAD under gradient conditions were calculated as described by Hartwick et al. [5]. Gradient delay volume was determined. Flow-rate was held constant at 1 ml/min. The ln k' (capacity factor) for each compound was obtained under isocratic conditions at five methanol concentrations from 0 to 10% methanol in 0.02 *M* ammonium dihydrogen phosphate, pH 5.6. The equation relating retention times to gradient slope was solved via a Fortran program.

Placental extracts

Placentas were obtained as soon as possible after delivery. The maternal surface was rapidly rinsed with 0.9% saline, blotted dry, and sub-surface

tissue of three to four intact lobules was quickly sampled with bone rongeurs cooled in liquid nitrogen. Frozen tissue pellets were stored at −80°C. Two or three tissue pellets from each placenta were combined and powdered under liquid nitrogen; the tissue powder (0.4—1.0 g) was extracted in 2 ml of ice-cold 12% trichloroacetic acid by vortexing for 30 s. Acid-insoluble material was sedimented by centrifugation at 11 000 *g* at 5°C for 20 min. Trichloroacetic acid was removed from the supernatant by vortexing with 4 ml of a 0.5 *M* solution of Alamine 336 in Freon 113 for 30 s [6]. Acid-soluble extracts were stored at −20°C until analysis by HPLC. Isolation of ribonucleosides from other acid-soluble components in placental extract was achieved by passage of an aliquot of the extract through an Affi-Gel-601 phenyl boronate column, followed by elution of the column with 0.1 *M* formic acid [7]; the eluate was evaporated to dryness in vacuo and reconstituted in water.

RESULTS

Optimization of chromatographic conditions

Chromatography of acid-soluble extracts of human term placenta on Varian MCH-10 columns was performed using linear gradient elution with methanol as the high-strength eluent and ammonium dihydrogen phosphate buffer as the low-strength eluent. Initial studies on an MCH-10 column with manufacturer-certified 5265 theoretical plates demonstrated that a gradient of 1% increase per min of methanol—water (60:43) in 0.05 *M* ammonium phosphate, pH 6.5, resolved the analytes of interest, i.e. adenosine, inosine and hypoxanthine from other UV-absorbing components. Order of elution was xanthine, hypoxanthine, inosine, β-NAD, adenosine, adenine. Retention time of adenosine was 27.4 min. Assignment of these six peaks was achieved by comparison of their retention times with retention times of standards and by spiking with standards. Ratios of absorbances at 250 nm to 260 nm were used to identify hypoxanthine, inosine, β-NAD and adenosine, and to confirm peak homogeneity of these analytes. Differences in 250/260 absorbance ratios of peaks in placental extracts compared to 250/260 absorbance ratios of the standards were < 3%; 250/260 absorbance ratios of the standards were: hypoxanthine 1.26, inosine 1.60, β-NAD 0.80 and adenosine 0.70. Peak purity of adenosine, inosine and hypoxanthine was confirmed by enzyme peak shift using, respectively, adenosine deaminase (Fig. 1), purine nucleoside phosphorylase (Fig. 2) and xanthine oxidase (Fig. 3). The identity of the β-NAD peak was confirmed in Affi-Gel 601 eluate which contained only the *cis*-diol components of placental extract; the putative β-NAD peak observed in the chromatogram of the eluate disappeared after NADase treatment of the eluate (results not shown). Simultaneous quantitation of hypoxanthine, inosine and adenosine was achieved from the one chromatogram on this column, but as the column aged, adequate resolution of hypoxanthine from xanthine and of inosine from β-NAD was not consistently observed, as indicated in Figs. 1 and 2 (panels A, B and C). Chromatography of placental extracts on a second Varian MCH-10 column (with 6620 theoretical plates) using the same mobile phase conditions, resulted in increased retention times, peak broadening and reversal of the order of elution of β-NAD and inosine. Fig. 3 shows the longer retention times of xanthine and

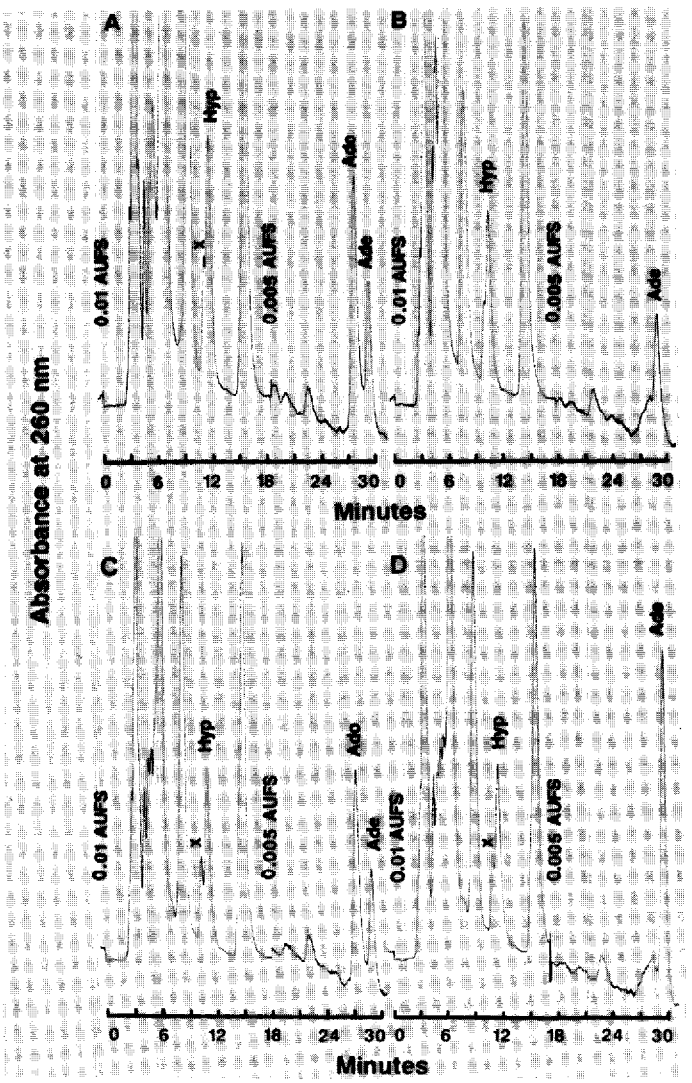

Fig. 1. Adenosine and adenine peak assignment in chromatograms of an acid-soluble extract of human term placenta, obtained on a Varian MCH-10 reversed-phase column. (A) Untreated extract. (B) Extract after treatment with adenosine deaminase. (C) Adenosine deaminase-treated extract (cf. B) spiked with adenosine (Ado). (D) Adenosine deaminase-treated extract (cf. B) spiked with adenine (Ade). Gradient elution: 1% increase per min of methanol—water (60:43) in 0.05 *M* ammonium dihydrogen phosphate, pH 6.5. Absorbance units full scale: 0.01 and 0.005, as indicated. Xanthine (X), t_R 10.7 ± 0.09 min, was incompletely resolved from hypoxanthine (Hyp), t_R 11.26 ± 0.13 min, and inosine co-eluted with β-NAD, t_R 14.85 + 0.47 min. Retention times are means ± S.D. of at least three determinations.

hypoxanthine (cf. Fig. 1) and confirmation of the assignments of xanthine and hypoxanthine peaks by xanthine oxidase peak shift. As indicated in Fig. 2 (panels D, E and F) a steeper linear gradient [2% increase per min of methanol—water (60:43) in 0.05 *M* ammonium dihydrogen phosphate (pH 6.5) for 20 min, followed by a 5% increase per min of high-strength eluent in the

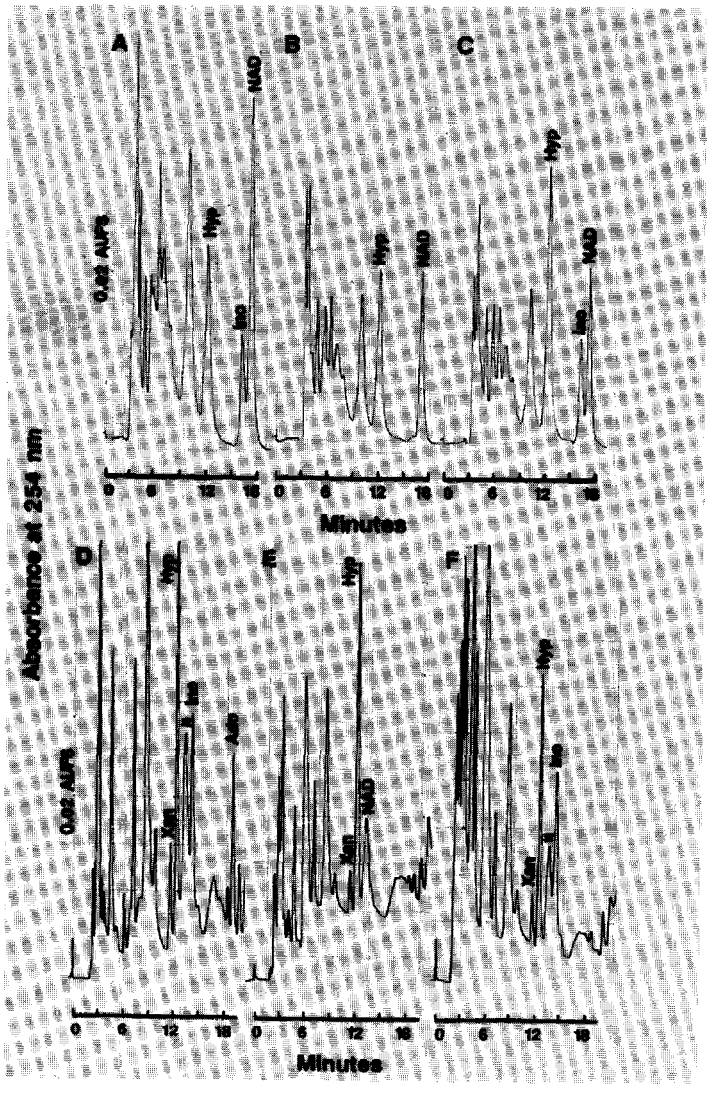

Fig. 2. Inosine peak assignment in chromatograms of acid-soluble extracts of human term placentas, obtained on different Varian MCH-10 reversed-phase columns. Extract, column and gradient elution for panels A, B and C, see Fig. 1. Chromatograms in panels D, E and F were obtained with a different extract on a different Varian MCH-10 reversed-phase column eluting with a 2% increase per min for 10 min followed by 10% increase per min of methanol—water (60:43) in 0.05 *M* ammonium dihydrogen phosphate pH 6.5. (A, D) Untreated extracts. (B, E) Same extracts treated with purine nucleoside phosphorylase. (C) Purine nucleoside phosphorylase-treated extract (cf. B) spiked with inosine. (F) Untreated extract (cf. D) treated with adenosine deaminase; adenosine (Ado) present in D was shifted. In panel E the Ado peak observed in D was lost as a result of adenosine deaminase present in commercial purine nucleoside phosphorylase. Absorbance units full scale: 0.02. Peaks: Ino = inosine; Xan = xanthine; Hyp = hypoxanthine; N (cf. D and F) = NAD.

ammonium dihydrogen phosphate buffer for 10 min] reduced retention times and sharpened the peaks so that quantitation could be achieved by peak-height measurement. However, the order of elution of inosine and β-NAD was not modified; peak assignment of inosine was confirmed by enzyme peak shift

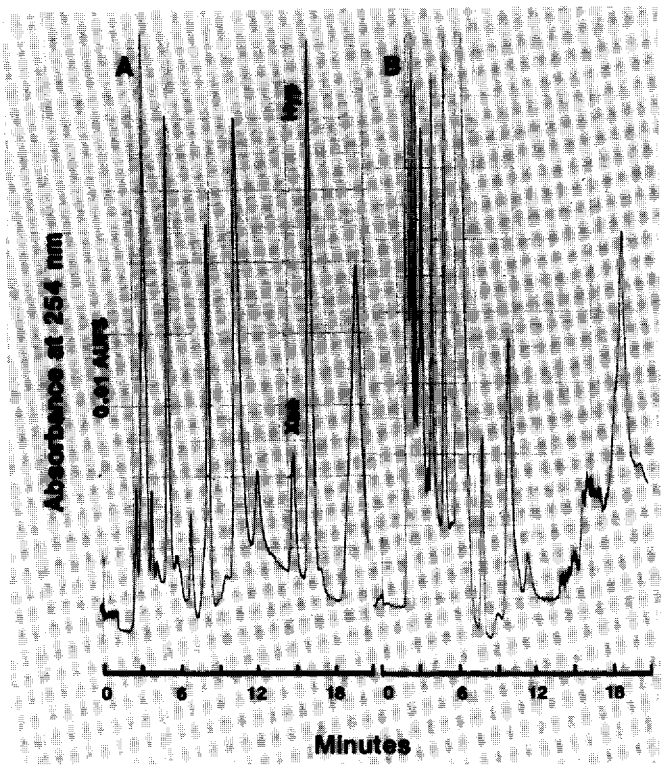

Fig. 3. Xanthine oxidase peak shift of xanthine (Xan) and hypoxanthine (Hyp) peaks in chromatograms of an acid-soluble extract of human term placenta on a Varian MCH-10 reversed-phase column. (A) Untreated extract. (B) Extract after treatment with xanthine oxidase. Same column as in Fig. 2 (panels D, E, F). Gradient elution: 1% increase per min of methanol—water (60:43) in 0.05 *M* ammonium dihydrogen phosphate, pH 6.5.

as shown in Fig. 2, panels D, E and F. The size of the β-NAD peak in the chromatogram in Fig. 2D was unusual; the β-NAD peak was generally very prominent and larger than the inosine peak, as shown in Fig. 2A. Use of a steeper gradient [3% increase per min of methanol—water (60:43) for 10 min, followed by a 10% increase per min for 10 min] resulted in further peak sharpening and elution of adenosine within 18 min without loss of resolution of hypoxanthine and inosine from xanthine and β-NAD, respectively (Fig. 4).

Gradient conditions which afforded the resolution shown in Fig. 4 were not satisfactory for separation on other Varian MCH-10 columns (with 6589 and 7001 theoretical plates) because inosine was not adequately resolved from the prominent β-NAD peak. Moreover, as inosine was normally the smaller peak, it could not be quantitated under these conditions. Partial resolution of standards of β-NAD and inosine on an octadecylsilica column with a mobile phase of 0.02 *M* potassium phosphate, pH 5.6, as the low-strength eluent, a 0.69% increase per min in methanol and a flow-rate of 1.5 ml/min has been reported [8]; in our hands, on Varian MCH-10 columns, these conditions did not resolve inosine and β-NAD, although a gradient slope of 1.4% increase per min in methanol with a flow-rate of 1.0 ml/min achieved adequate resolution of inosine and β-NAD. Optimization of these gradient conditions in order to

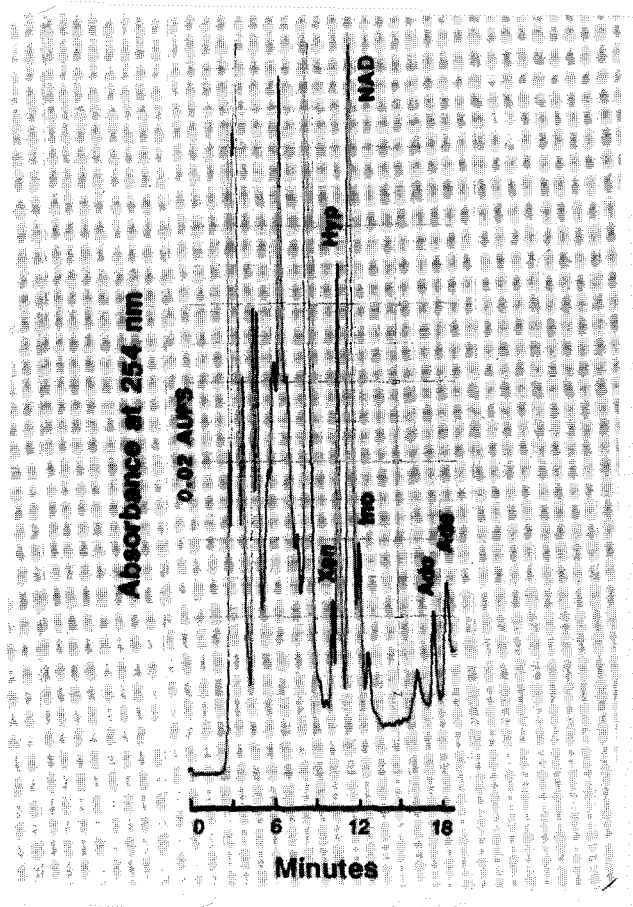

Fig. 4. Typical chromatogram of acid-soluble extract of human term placenta. Same column as in Fig. 2 (panels D, E and F). Gradient elution: 3% increase per min for 10 min, followed by 10% increase per min of methanol—water (60:43) in ammonium dihydrogen phosphate, pH 6.5. For abbreviations see legends to Figs. 1 and 2.

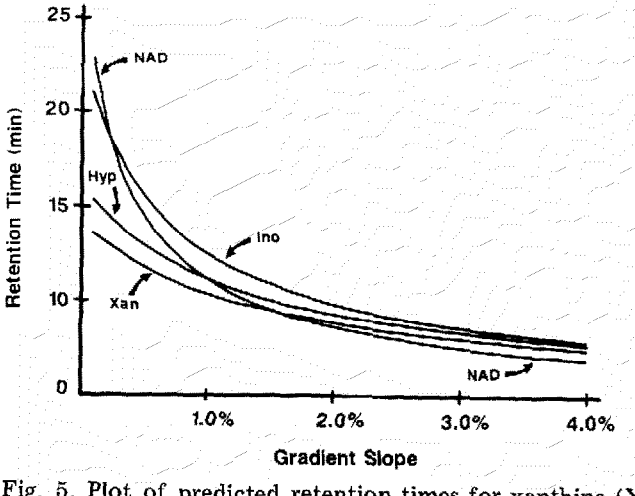

Fig. 5. Plot of predicted retention times for xanthine (Xan), hypoxanthine (Hyp), β-NAD (NAD) and inosine (Ino) versus the gradient slope, %/min change in methanol. Flow-rate, gradient delay and initial methanol concentration, i.e. 0% methanol, were held constant and retention times were calculated for various gradient slopes using a Fortran program.

resolve xanthine, hypoxanthine, β-NAD and inosine simultaneously was investigated, as simultaneous resolution would allow quantitation of hypoxanthine and inosine from the same chromatogram. For this purpose the equations reported by Hartwick et al. [5] to predict retention times and gradient conditions required to separate nucleosides and bases were used. Correlation coefficients for regression analysis of the relationships between ln k' and percentage methanol in the mobile phase for each of the four compounds were at least −0.99, indicating that the relationships were indeed linear, as reported for xanthine, hypoxanthine and inosine by Hartwick et al. [5]. As indicated in Fig. 5, the optimal gradient slope for separation of inosine and β-NAD was predicted to be 1.3%, and the predicted retention times showed that xanthine, hypoxanthine, inosine and β-NAD could not be resolved simultaneously during a single elution. Thus, on the columns with a higher number of manufacturer-specified theoretical plates, a linear gradient of 2% increase per min in methanol–water (70:33) in 0.02 *M* ammonium dihydrogen phosphate, pH 5.6, was used for resolution of inosine from β-NAD and quantitation of inosine, and adenosine and hypoxanthine were quantitated from a separate chromatogram using the two-step gradient with pH 6.5 buffer as the low-strength eluent described above and in the legend to Fig. 4.

Percentage recovery

Recoveries of adenosine, inosine and hypoxanthine from solution in trichloroacetic acid were determined by HPLC. Trichloroacetic acid solutions of known amounts of the compounds were extracted with Alamine 336 in Freon 113, as described in Experimental, and chromatographed. With the exception

TABLE I

RECOVERY OF ADENOSINE, INOSINE AND HYPOXANTHINE STANDARDS FROM SOLUTION IN TRICHLOROACETIC ACID

Trichloroacetic acid was extracted with 0.5 *M* Alamine 336 in Freon 113, as described in Experimental; HPLC analysis of the extracted solutions was performed in triplicate.

Concentration of standards in trichloroacetic acid (nmol/ml)		Recovery (%)		
		Adenosine	Inosine	Hypoxanthine
22.2	Mean	90.59	99.95	91.70
	S.D.	4.33	1.73	1.99
	R.S.D.*	0.05	0.02	0.02
44.4	Mean	91.34	100.52	93.20
	S.D.	7.29	1.30	2.16
	R.S.D.*	0.08	0.01	0.02
88.9	Mean	85.64	99.84	89.96
	S.D.	2.57	1.81	2.99
	R.S.D.*	0.03	0.02	0.03

*R.S.D. (relative standard deviation) = S.D./mean.

of the recovery of 88.9 nmol of adenosine per ml of acid solution, which was 85.64%, recoveries of the three compounds over the range of 22.2—88.9 nmol/ml were > 90% (Table I). Concentrations of adenosine determined in the acid-soluble placental extracts were less than 30 nmol/ml. Recoveries were also determined radiometrically; ^{14}C-labeled standards were added to the suspension of powdered tissue in trichloroacetic acid prior to vortexing and extraction with Alamine 336 in Freon 113. Experiments were performed in triplicate. Recoveries were greater than 90%: 92.5 ± 3.3% for adenosine, 91.8 ± 3.6% for inosine and 93.0 ± 1.4% for hypoxanthine (means ± S.D.).

Measurement of placental adenosine, inosine and hypoxanthine

Adenosine, inosine and hypoxanthine in acid-soluble extracts of eight placentas were quantitated using optimized conditions of gradient elution described above. Findings (means ± S.D.) are summarized in Table II as nmol/g of placenta; standard deviations were less than 5% of the mean values. The mode of delivery of the infant is noted. With one exception, the preeclamptic placenta, all placentas were from uncomplicated pregnancies. Adenosine levels

TABLE II

ADENOSINE, INOSINE AND HYPOXANTHINE LEVELS IN HUMAN TERM PLACENTA

Delivery type		Level* (nmol/g of placenta)		
		Adenosine	Inosine	Hypoxanthine
Spontaneous vaginal	Mean	22.84	63.17	200.6
	S.D.	0.78	1.24	
Spontaneous vaginal	Mean	22.56	95.39	336.63
	S.D.	0.57	5.21	3.18
Spontaneous vaginal	Mean	41.11	53.68	79.51
	S.D.	0.59	1.73	1.01
Spontaneous vaginal**	Mean	99.55	86.28	142.89
	S.D.	2.06	8.49	1.56
Induced vaginal	Mean	56.59	101.72	218.39
	S.D.	0.76	0.72	2.50
Caesarian section after induction	Mean	51.38	81.98	211.62
	S.D.	0.28	0.61	2.89
Caesarian section after induction	Mean	22.49	45.35	458.13
	S.D.	0.56	0.56	19.93
Casarian section	Mean	16.53	34.21	88.59
	S.D.	0.68	1.51	3.64

*Values are mean ± S.D. of three HPLC determinations, or mean of two HPLC determinations, of an acid-soluble extract of combined samples from two to three lobules of a single placenta.

**Preeclamptic placenta; sample extracted was from one lobule.

in the normal placentas ranged from 16.53 ± 0.68 to 56.59 ± 0.76 nmol/g and were lower than inosine and hypoxanthine levels. In contrast, the adenosine content of the preeclamptic placenta, 99.55 ± 2.06 nmol/g, was significantly higher than levels in the placentas from normal pregnancies and was moreover higher than the inosine content of this placenta.

DISCUSSION

This study describes conditions for precise determination of levels of adenosine, inosine and hypoxanthine in human term placenta. Measurement of these analytes by reversed-phase HPLC of acid-soluble extracts of placenta was achieved on octadecylsilica columns using gradient conditions of elution which optimized resolution of the analytes from other UV-absorbing components of the extracts. The study used only Varian MCH-10 octadecylsilica columns in order to ensure consistent column-to-column chromatographic characteristics. Even so, variability amongst the columns was observed, evidenced by reversal of the order of elution of β-NAD and inosine and by increase in retention times, particularly of the later-eluting peaks such as adenosine and adenine. Simple modification of the linear gradient slope to increase the rate of increase in organic modifier sufficed to reduce the retention times and sharpen the adenosine peak, thus achieving quantitation of adenosine and hypoxanthine with the same mobile phase. However, on some Varian MCH-10 columns, these mobile phase conditions did not resolve β-NAD from inosine, and it was shown using the relationship between predicted retention times and gradient slope [6] that separation of β-NAD and inosine could be optimized at a lower pH and with a different gradient. Solvophobic [9] mechanisms are believed to be primarily responsible for retention of nucleosides and bases on reversed-phase columns, but evidence has suggested that retention of charged species such as nucleotides may also involve binding to free silanols resulting in mixed-mode separation [10—12]. Columns which gave reduced retention times for β-NAD may have differed in having fewer residual free silanols interacting with β-NAD.

The gradient conditions developed for resolution and quantitation of adenosine, inosine and hypoxanthine on Varian MCH-10 reversed-phase columns also resolved xanthine, β-NAD and adenine. Quantitation of these purine compounds would be possible under the conditions described but was not the focus of these studies. Nevertheless it is noteworthy that β-NAD was generally a very prominent component of the placental extracts and that adenine was present in all extracts analyzed. Although adenine is present in urine, it is not usually found in tissues or body fluids [13].

The mean contents of adenosine and inosine found in representative samples from the submaternal surface of the seven placentas from normal deliveries were 30.6 ± 11.5 nmol/g and 68.0 ± 25.8 nmol/g, respectively. These values are four- to seven-fold lower than those obtained in the earlier study [1] in which 20—25 g of placental tissue was extracted in water at 90—95° C for subsequent paper chromatographic isolation of the compounds. The difference can be ascribed to the time needed to achieve heat denaturation of adenine nucleotide-degrading enzymes, particularly heat-stable AMP phosphatase which is highly active in the human term placenta [14]. The freeze-clamping and acid extrac-

tion techniques used in the present study ensured immediate inactivation of enzymes and stabilization of nucleotide, nucleoside and nucleobase pools. In all the placentas except that from the preeclamptic pregnancy, inosine levels were higher than adenosine levels and lower than hypoxanthine levels, suggesting altered adenosine metabolism in the preeclamptic placenta. Moreover, the hypoxanthine content showed great variation amongst the seven placentas from normal pregnancies. These aspects of the study have been further investigated using the analytical techniques described here and will be reported elsewhere [15].

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