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# Measurement of hypoxanthine and xanthine in late-gestation human amniotic fluid by reversed-phase high-performance liquid chromatography with photodiode-array detection

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## ABSTRACT

A robust analytical method was developed for measurement of hypoxanthine and xanthine in late-gestation human amniotic fluid by reversed-phase high-performance liquid chromatography using diode-array detection. Purity of analyte peaks was confirmed via on-line analysis of peak spectra utilizing the purity parameter treatment of spectral data. Amniotic fluid obtained by amniocentesis was deproteinized by centrifugal ultrafiltration and chromatographed on an octadecylsilica column using isocratic elution with 1% (v/v) acetonitrile in 0.05 *M* ammonium dihydrogenphosphate pH 6.0, hypoxanthine and xanthine were resolved, but the hypoxanthine peak was not pure. Chromatography on a column of polar end-capped octadecylsilica, using similar mobile phase conditions, yielded spectrally pure peaks of hypoxanthine and xanthine. Hypoxanthine and xanthine levels in amniotic fluid from fourteen patients, gestational age 34–39 weeks, ranged from 0.56 to 2.74 $\mu M$ and 1.62 to 5.52 $\mu M$, respectively.

## INTRODUCTION

During hypoxia and ischemia, hypoxanthine (Hyp) and xanthine (Xan), the major metabolites of degradation of energy-rich purine nucleotides, accumulate in extracellular body fluids such as plasma, urine and cerebrospinal fluid [1–4]. Hyp, formed by breakdown of ATP, accumulates because further metabolism, such as salvage to IMP or oxidation to Xan by xanthine oxidase, is inhibited under hypoxic conditions. Similarly, under hypoxic conditions salvage of guanine formed by guanine nucleotide breakdown is inhibited; as a result guanine can be hydrolyzed to Xan via the action of guanase [5]. The concentration of Hyp in extracellular fluids has been proposed as a sensitive indicator of hypoxia and its metabolic sequelae in tissues [4]. Attention has been given to ascertaining whether Hyp may be useful as an indicator of fetal hypoxia, measuring Hyp levels in

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amniotic fluid at delivery [6] However, there have been no studies examining a possible relationship between late-gestation amniotic fluid Hyp and *in utero* hypoxia. *In utero* hypoxia results in significant perinatal morbidity and mortality. A biochemical marker for hypoxia, such as elevated amniotic fluid Hyp, could be helpful in clinical management. Late-gestation amniotic fluid is frequently available as a result of amniocentesis performed to determine fetal lung maturity, so that studies of the clinical potential of Hyp as an indicator of *in utero* hypoxia are feasible. Before such studies can be undertaken, validated methods for analysis of amniotic fluid Hyp are required. In particular, sample preparation should be optimized and a robust method of reversed-phase high-performance liquid chromatography (HPLC) developed, in which Hyp and Xan are well resolved.

We report here optimized methods for HPLC analysis of Hyp and Xan in amniotic fluid with use of photodiode-array detection and on-line validation of the purity of analyte peaks in each chromatogram. We report also baseline levels of these analytes in late-gestation amniotic fluid obtained when amniocentesis was performed in patients who presented no clinical evidence of *in utero* hypoxia.

## EXPERIMENTAL

### *Materials*

Acetonitrile, methanol and orthophosphoric acid were HPLC-grade from Fisher Scientific (Springfield, NJ, U.S.A.). Ammonium dihydrogen phosphate was Baker-analyzed grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Hyp and uridine were obtained from Pharmacia P.-L. Biochemicals (Piscataway, NJ, U.S.A.). Xan, uric acid, creatinine, lithium nitrate and xanthine oxidase (specific activity 1.2 U/mg of protein) were obtained from Sigma (St. Louis, MO, U.S.A.). Nylon Acrodisc 0.45-$\mu$m disposable membrane filters were supplied by Gelman Sciences (Ann Arbor, MI, U.S.A.) and Centricon-10 microconcentrators (10 000 MW cut-off membrane) by Amicon (Danvers, MA, U.S.A.). Distilled water was passed through an IWT charcoal cartridge and redistilled in an all-glass system.

### *Sample preparation*

Amniotic fluid was obtained at or near term and prior to onset of labor *via* transabdominal amniocentesis performed for clinical indications. Amniotic fluid was drawn into a syringe using a twenty-gauge needle; approximately 5 ml were immediately centrifuged at 1700 *g* for 10 min to remove any particulate matter, and either processed for HPLC or stored at $-20$°C for up to two weeks until processing. Samples were generally free from blood; any samples contaminated with blood were discarded. Amniotic fluid was deproteinized by ultracentrifugation through Centricon-10 microconcentrators (10 000 MW cut-off membrane) at 4300 *g* at 10°C for 180 min. Ultrafiltered samples were analyzed immediately or stored at $-20$°C until HPLC analysis within one week. Amniotic fluid was also extracted by acid treatment, using either 0.5 volume of 4 *M* perchloric acid fol-

lowed by neutralization with 0.5 volume of 4 *M* potassium carbonate, or 1 volume of ice-cold 24% (w/v) trichloroacetic acid which was then extracted with a 0.5 *M* solution of Alamine 336 in Freon 113 [7,8]. Acid-soluble extracts were stored at −20°C until analysis by HPLC.

*Instrumentation*

HPLC was performed using the following Varian components: 5560 chromatograph, column heater, 8085 autosampler and 9060 diode-array detector. Injection was performed manually or automatically via a Rheodyne 7126 valve injector, using 20- or 50-μl loops. Chromatographic profiles, peak retention times and integrated peak areas were recorded with a Spectra-Physics 4270 data integrator. Spectral data generated by the diode-array detector were transferred to a computer and analyzed by Polysoc software to display and overlay spectra and compute purity parameters.

*Chromatography*

HPLC columns packed with spherical octadecylsilica were supplied by Keystone Scientific (State College, PA, U.S.A.). Packing, particle size and column dimensions were as follows: Shandon $C_{18}$ Hypersil, 3 μm, 150 mm × 4.6 mm I.D.; Shandon $C_{18}$ Hypersil, 5 μm, 250 mm × 4.6 mm I.D.; and Keystone ODS/A, 5 μm, 250 mm × 4.6 mm I.D. Brownlee 15 mm × 3.2 mm I.D. guard cartridges packed with sperical 7-μm wide-pore octadecylsilica were used routinely.

Mobile phases contained ammonium dihydrogenphosphate buffer as the low-strength eluent and acetonitrile as the high-strength eluent. Buffer pH was adjusted with potassium hydroxide or orthophosphoric acid, and solutions were filtered through a 0.45-μm Millipore membrane. Mobile phases were degassed by sonication.

Capacity factors ($k' = (t_R - t_0)/t_0$) were determined from replicate measurements of analyte retention times ($t_R$); void volume ($t_0$) was determined with lithium nitrate. Resolution, $R_S$, was determined from the equation $R_S = (1/4)(\alpha - 1)N^{0.5}[k'/(1 + k')]$, where $N$ is the column plate number and $\alpha$ is the separation factor [9].

Peaks of Hyp and Xan in chromatograms of amniotic fluid extracts were identified by comparison of retention times with those of standards, by 'spiking' with standards, by enzymatic peak-shift [8] and by comparison of peak apex purity parameters with those of standards under the same mobile phase conditions. Enzymatic peak-shift was performed by addition of 0.3 U of xanthine oxidase (EC 1.2.3.2) to a mixture of 400 μl of amniotic fluid extract and 400 μl of 0.1 *M* phosphate buffer pH 7.4; assays were incubated at 37°C for 30 min Reaction was stopped by heating at 100°C for 3 min. Samples were filtered through a 0.45-μm filter and stored at −20°C until analysis by HPLC.

*Purity parameters*

Purity parameters of Hyp and Xan peaks were obtained routinely for confirmation of peak purity in chromatograms of amniotic fluid extracts. The purity parameter treatment of spectral data of a peak employs a weighting factor, the square of the absorbance, to reduce the data to a single value, the purity parameter; this numerical value represents the characteristic average wavelength of a spectrum [10]. Purity parameters computed from spectra of the upslope, apex and downslope of a pure chromatographic peak are identical. Comparison of purity parameters of sample peaks with those of the peaks of standards can be used to confirm sample peak identity [10,11]. The 9060 diode-array detector computes peak upslope, apex and downslope purity parameters over a single designated wavelength range. With Polysoc software, processing of spectral data from a peak can be performed over more than one wavelength range, and an optimized wavelength range for computation of purity parameters can be determined. Use of an optimized wavelength range for measurement of purity parameters is advantageous when signal-to-noise ratios are low. In this study optimized wavelength windows used for Hyp and Xan were 229–268 and 249–287 nm, respectively. A sample peak was initially evaluated as pure when the peak apex purity parameter agreed to $\pm$ 1 nm with that of the standard determined under identical mobile phase conditions. Purity of the peak was confirmed when upslope, apex and downslope purity parameters agreed with a relative standard deviation (R.S.D.) $<0.5\%$.

*Quantitation*

Quantitation of Hyp and Xan in amniotic fluid extracts was performed from calibration curves which related integrated peak areas to the concentration of Hyp and Xan standards in a 50-$\mu$l injection volume. Amniotic fluid extracts were routinely analyzed in duplicate.

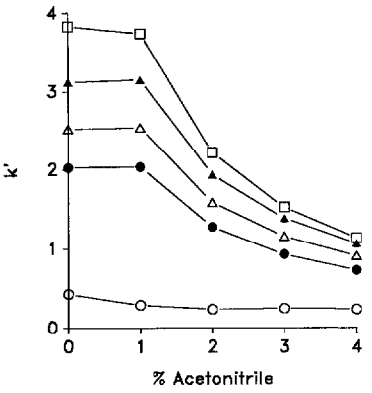
*Clinical information*

Clinical information obtained includes any medical diagnoses complicating pregnancy. Also, at birth, Apgar scores, umbilical cord acid–base determination and weight were used to assess newborn well being.

## RESULTS

*Method development*

Our previous studies showed Hyp and Xan to be well resolved on a 150 mm $\times$ 4.6 mm I.D., 3-$\mu$m $C_{18}$ Hypersil column with a shallow gradient of acetonitrile in an ammonium dihydrogenphosphate buffer pH 6.0 [11] Using similar, but isocratic conditons the effect of percentage acetonitrile on capacity factors of the two purines and three other putative components of amniotic fluid showed that resolution of all five compounds was optimal at 1–2% acetonitrile (Fig. 1). The



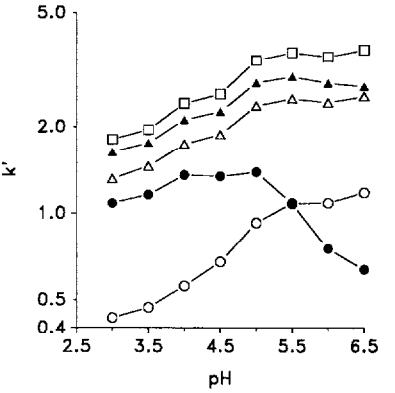




Fig 1. Plot of capacity factor *versus* percentage (v/v) acetonitrile in the mobile phase for creatinine (○), uric acid (●), hypoxanthine (△), xanthine (▲) and uridine (□) Low-strength eluent, 0 05 *M* ammonium dihydrogenphosphate pH 6 0 Column, 150 mm × 4.6 mm I D , 3 μm average particle size, $C_{18}$ Hypersil. Column temperature, 40°C. Flow-rate, 1 0 ml/min Injection volume, 20 μl Detection wavelength, 254 nm.

Fig 2 Plot of capacity factor *versus* pH of the mobile phase for creatinine (○), uric acid (●), hypoxanthine (△), xanthine (▲) and uridine (□) Eluent, 0.05 *M* ammonium dihydrogenphosphate, adjusted to the pH indicated, containing 1% (v/v) acetonitrile Column, 150 mm × 4 6 mm I.D , 3 μm average particle size, $C_{18}$ Hypersil Column temperature, 40°C Flow-rate, 1 0 ml/min Injection volume, 20 μl. Detection wavelength, 254 nm

effect of buffer pH on capacity factors at 1% acetonitrile showed that resolution of Hyp, Xan and uridine was optimal at pH 5–6, and that in this pH range uric acid and creatinine would not interfere with the Hyp peak (Fig. 2). Further method development was performed with buffer pH 6.0, as this pH was closer to that of amniotic fluid. Fig. 3A shows a typical chromatogram of ultrafiltered amniotic fluid under these conditions. Although Hyp and Xan peaks were sharp and mutually well resolved, Hyp eluted on the trailing edge of a major unidentified peak, and xanthine oxidase peak-shift showed that the Hyp peak was not homogeneous. A longer column with similar reversed-phase packing, 5-μm $C_{18}$ Hypersil, achieved apparent resolution of the two purines from other major UV-absorbing components of amniotic fluid (Fig. 3B). However, the profile of the leading edge of the Hyp peak suggested the presence of a co-eluting contaminant, and this was confirmed by the peak apex purity parameter which was not within the acceptable range.

Resolution of amniotic fluid Hyp from unknown co-elutants was attempted using a 250-mm-long Keystone ODS/A column. ODS/A is a modified octadecylsilica, in which the end-capping group has polar character. Particle and pore sizes of the ODS/A packing are the same as those of 5-μm Hypersil, but the former has greater carbon loading than Hypersil. Using a mobile phase flow-rate of 0.9 ml/min, a column temperature of 40°C and Hyp as the solute, the efficiency of the 250-mm-long Keystone ODS/A column was determined to be somewhat lower than that of the 250-mm-long $C_{18}$ Hypersil ($N_{\text{Hypersil}}$ = 13 500 *versus*

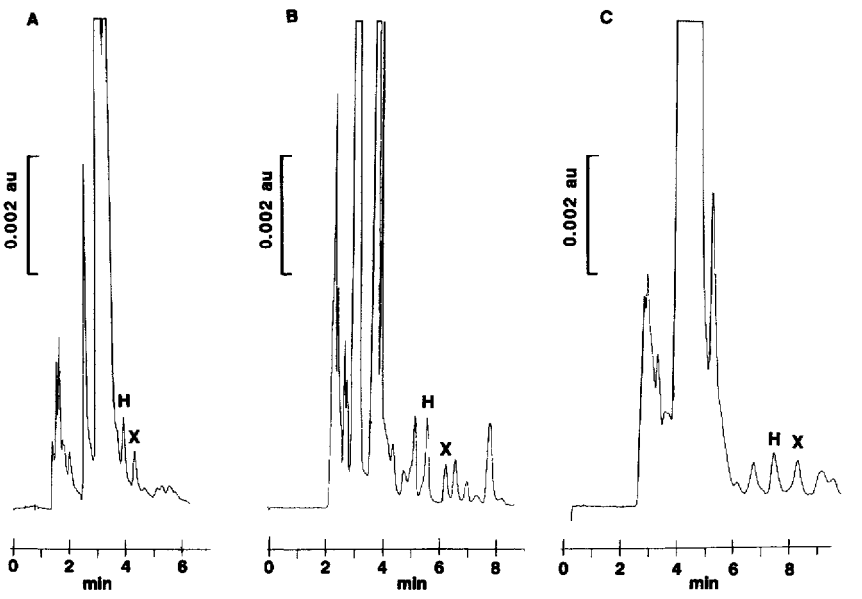



Fig. 3. Chromatograms of the same amniotic fluid extract on three different columns Mobile phase, 1% (v/v) acetonitrile in 0.05 *M* ammonium dihydrogenphosphate pH 6.0 Flow-rate, 1 0 ml/min Detection wavelength, 254 nm Chart speed, 1 cm/min. (A) Column, 150 mm × 4 6 mm I D , 3 $\mu$m average particle size, $C_{18}$ Hypersil, column temperature, 40°C, injection volume, 20 $\mu$l (B) Column, 250 mm × 4.6 mm I.D , 5 $\mu$m average particle size, $C_{18}$ Hypersil, column temperature 40°C, injection volume, 20 $\mu$l (C) Column, 250 mm × 4.6 mm I D., 5 $\mu$m average particle size, ODS/A Keystone, column temperature, 45°C, injection volume, 50 $\mu$l Peaks. H = hypoxanthine, X = xanthine

$N_{\text{Keystone}}$ = 12 000) but actual resolution of Hyp and Xan was improved ($R_{S\ \text{Hypersil}}$ = 2.3 *versus* $R_{S\ \text{Keystone}}$ = 3.2). The ODS/A column achieved baseline resolution of amniotic fluid Hyp and Xan as indicated in Fig. 3C and Fig. 4A. Xanthine oxidase peak-shift demonstrated that Hyp and Xan peaks were free from co-eluting UV-absorbing contaminants (Figs. 4A and B), and, furthermore, peak apex purity parameters of the peaks of both purines agreed with those of standards, and upslope, apex and downslope purity parameters were concordant, indicating that the peaks were pure. Sample injection volume was increased to 50 $\mu$l, resulting in improved signal-to-noise ratio for analyte peaks. Column temperature was optimized to 45°C; at 50°C analyte peaks were sharper, but retention times were shorter and resolution was reduced. In Fig. 4A and B, the peak with retention time 10.0 min is uridine, as indicated by retention time and spectrum of a standard uridine peak.

*Optimized chromatographic method*

HPLC of 50-$\mu$l samples of centrifugally ultrafiltered amniotic fluid was performed on a 250 mm × 4.6 mm I.D. Keystone ODS/A column at a column temperature of 45°C, using 10 min isocratic elution at 1.0 ml/min with 1% (v/v)

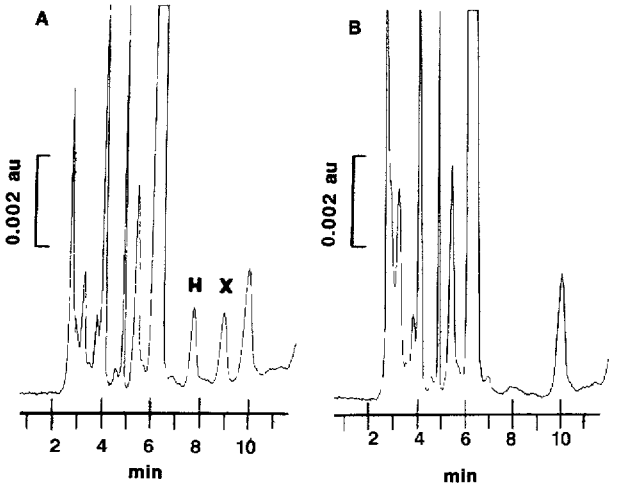



Fig 4 Xanthine oxidase peak-shift of hypoxanthine and xanthine peaks in an amniotic fluid extract. Chromatograms were obtained on a 250 mm × 4.6 mm I D., 5 $\mu$m average particle size, ODS/A Keystone column (No. 2) using isocratic elution with 1% (v/v) acetonitrile in 0 05 *M* ammonium dihydrogenphosphate pH 6 0. Flow-rate, 1 0 ml/min Column temperature, 45°C Injection volume, 50 $\mu$l Detection wavelength, 254 nm Chart speed, 1 cm/min. (A) Untreated extract, (B) extract after treatment with xanthine oxidase. The peak of $t_R$ 10 0 min is uridine Peaks: H = hypoxanthine, X = xanthine

acetonitrile in 0.05 *M* ammonium dihydrogenphosphate pH 6.0, followed by a column clean-up cycle, as follows: 10–12 min, 1–50% acetonitrile; 12–17 min, 50% acetonitrile; 17–18 min, 50–1% acetonitrile; 18–19 min, flow-rate changed to 2.0 ml/min; 19–24 min, re-equilibration with the isocratic mobile phase at 2.0 ml/min; 24–25 min, flow-rate changed to 1.0 ml/min; 25–30 min, re-equilibration with the isocratic mobile phase at 1.0 ml/min. Total run time was normally 30 min. However, the time required for column re-equilibration increased after approximately 90 injections of amniotic fluid extracts. Apex, upslope and downslope purity parameters of analyte peaks were routinely recorded for each chromatogram. Apex purity parameters of standards are shown in Table I. Fig. 5 compares typical sample peak apex spectra with spectra of the peaks of standards.

In chromatograms of amniotic fluid extracts from four different patients the Xan peak was obscured by a prominent unidentified peak (Fig. 6A) which had a $\lambda_{max}$ of 244 nm. Reduction of mobile phase pH to 4.5 shifted the unknown peak to longer retention time and yielded a pure Xan peak (Fig. 6B). However, at pH 4.5 the Hyp peak was not pure, and analysis at this pH was employed only for quantitation of Xan when the contaminant co-eluting with Xan at pH 6.0 was present. The contaminant itself was pure at pH 4.5, with concordant upslope, apex and downslope purity parameters, but was not identified. It was possibly a dietary metabolite.

TABLE I

PEAK APEX PURITY PARAMETERS OF HYPOXANTHINE AND XANTHINE STANDARDS

Means ± S D of twelve observations at 1 25–10 $\mu M$ hypoxanthine and xanthine

| Compound | Mobile phase pH | Wavelength range (nm) | Purity parameter (nm) |
|---|---|---|---|
| Hypoxanthine | 6 0 | 229–268 | 249 43 ± 0 04 |
| Xanthine | 6 0 | 249–287 | 266 18 ± 0 07 |
| Xanthine | 4 5 | 249–287 | 266 09 ± 0 05 |

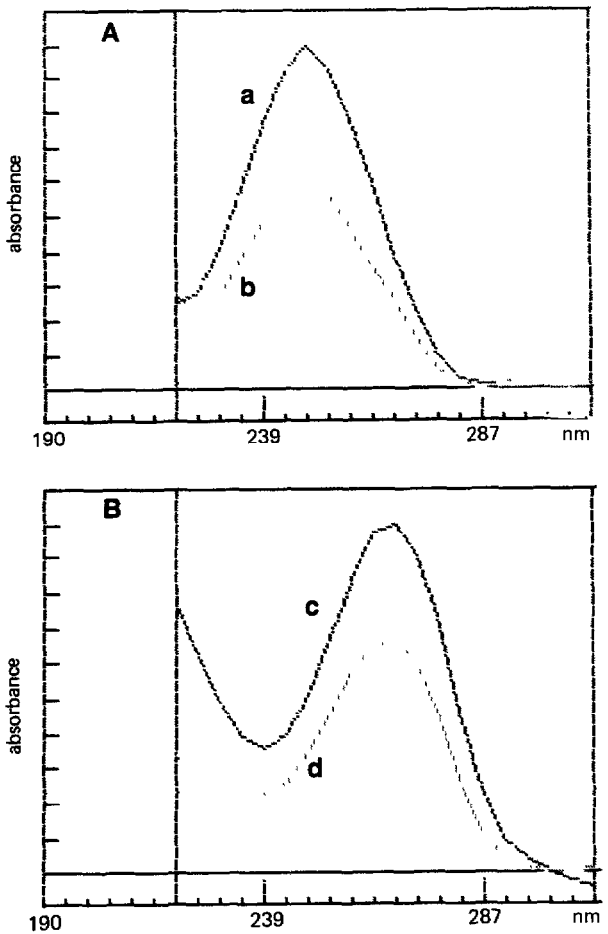



Fig 5. Peak apex spectra (A) Hypoxanthine, (B) xanthine Spectra of chromatographic peaks of standards of hypoxanthine (a) and xanthine (d), typical spectra of chromatographic peaks of hypoxanthine (b) and xanthine (c) in amniotic fluid extracts Spectra displayed via Polysoc software Conditions of chromatography as described in legend to Fig 4

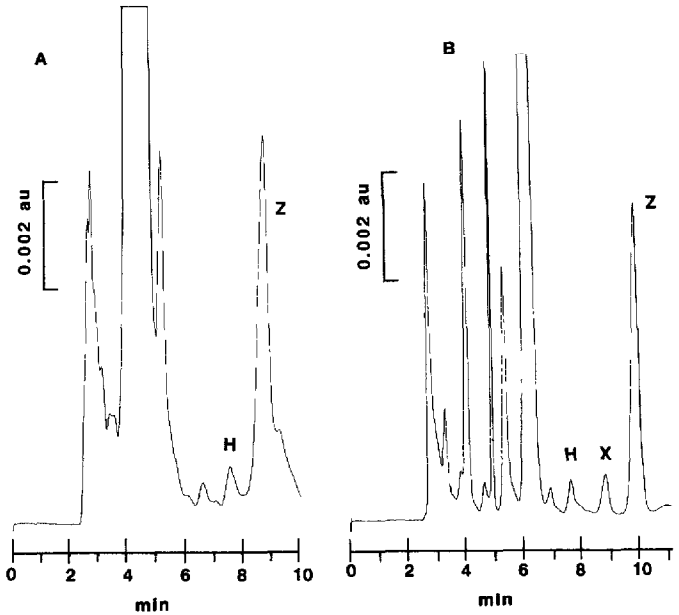



Fig 6 Chromatogams of an amniotic fluid sample at mobile phase pH 6 0 and 4 5 For other chromatographic conditions see legend to Fig 4 (A) Mobile phase pH 6.0; xanthine is obscured by co-eluting contaminant, Z (B) Mobile phase pH 4 5; the contaminant is shifted to $t_R$ 10 0 min, and the xanthine peak (X) is pure Peak H = hypoxanthine.

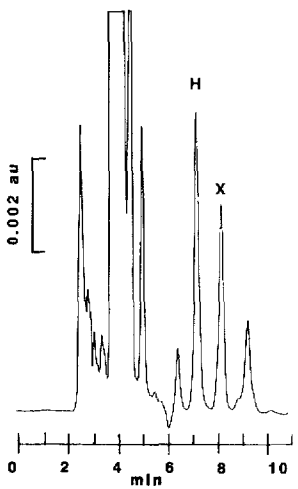



Fig 7 Chromatogram of the extract of amniotic fluid of patient gestational age 41 weeks with oligohydramnios Amniotic fluid sample was obtained via an intra-uterine catheter prior to onset of labor Column, 250 mm × 4.6 mm I.D , 5 μm average particle size, Keystone ODS/A No 3. For the chromatographic conditions see legend to Fig 4 Chart speed, 1 cm/min. Apex purity parameters for hypoxanthine (H) and xanthine (X) were 249.2 and 266 1 nm, respectively Upslope, apex and downslope purity parameters of both peaks were concordant with R S.D. < 0.1%.

Keystone ODS/A is a new form of octadecylsilica. Three different Keystone ODS/A columns were used in this study; the second (No. 2) and third (No. 3) columns were packed with the same batch of ODS/A silica. Analyses reported here were performed for the most part on the second column. The third column was significantly more efficient than the second, as evidenced by sharper peaks of the analytes (*cf.* Fig. 4A and Fig. 7), increased $N$ for Hyp (under optimized chromatographic conditions, $N_{\text{Keystone}}$ No. 2 = 9500 *versus* $N_{\text{Keystone}}$ No. 3 = 18 500), and improved resolution of the analytes ($R_S$ Keystone No. 2 = 4.0 *versus* $R_S$ Keystone No. 3 = 4.7). Improved efficiency of the third column was most likely due to improved techniques of packing used by the manufacturer [12]. Other chromatographic characteristics of the two columns were identical, as evidenced by comparison of chromatograms of the same extract on each column (results not shown).

*Sample preparation*

Extracts of amniotic fluid prepared by acidification with trichloroacetic acid followed by removal of the acid with Alamine 336 contained artefactual peaks, including a prominent peak with retention time 6.5 min which interfered with the Hyp peak (results not shown). Preparation of amniotic fluid extract by acidification with perchloric acid and subsequent neutralization resulted in sample dilution and analyte recoveries of less than 65%. Deproteinizing amniotic fluid by centrifugal ultrafiltration was the sample preparation method of choice; the sample was not diluted and the chromatogram was free of artefactual peaks.

*Recovery*

Recovery of Hyp and Xan from amniotic fluid was determined by spiking amniotic fluid samples with 1, 2, 3 and 5 $\mu M$ standards. Spiked and unspiked samples were deproteinized by centrifugal ultrafiltration and analyzed by HPLC as described above. Concentrations of Hyp and Xan were obtained from calibration curves. Recoveries of Hyp and Xan, calculated from the difference between paired spiked and unspiked samples, were 101.2 ± 3.7 and 99.8 ± 7.3% (mean ± S.D., $n$ = 9), respectively.

*Quantitation of hypoxanthine and xanthine in amniotic fluid*

Calibration curves for Hyp were linear from 0.1 to 20 $\mu M$. Linear regression analysis of the dependence of peak area ($y$) on concentrations of the standards ($x$) gave equations and correlation coefficients as follows: 0.1–1.0 $\mu M$, $y = 18075x - 691.7$ ($r$ = 0.999); 1.0–5.0 $\mu M$, $y = 18526x + 478.1$ ($r$ = 0.999); 2.5–20 $\mu M$, $y = 19921x - 1994.7$ ($r$ = 0.999). Calibration curves for Xan at pH 6.0 and 4.5 were identical and linear from 2.5 to 20 $\mu M$ ($y = 11827x - 2665.8$, $r$ = 0.999). Detection limits for Hyp and Xan were 60 and 90 n$M$, respectively (signal-to-noise ratio, 2:1). Levels of the purines were determined in amniotic fluid from fourteen patients. Results, means of duplicate measurements, are shown in Table

II, together with gestational age of the patients and the maternal diagnosis which prompted the determination of fetal lung maturity. All newborn weights were appropriate for gestational age and no evidence of *in utero* hypoxia was apparent as indicated by normal Apgar scores and acid–base values. The range of all Hyp levels measured was 0.56–2.74 $\mu M$. Mean (± S.D.) values at 37, 38 and 39 weeks gestation were 0.75 ± 0.17 $\mu M$ ($n$ = 3), 1.28 ± 0.35 $\mu M$ ($n$ = 3) and 1.54 ± 0.28 $\mu M$ ($n$ = 3), respectively. Of the Hyp levels measured in amniotic fluid from five patients at 36 weeks of gestation, four had a mean (± S.D.) value of 0.82 ± 0.13 $\mu M$; the fifth, 2.74 $\mu M$, was significantly higher. Amniotic fluid from patient No. 6 was analyzed at 36 and 37 weeks; levels of Hyp, 0.98 and 0.90 $\mu M$, were similar at these periods, as were levels of Xan (Table II). Xan levels were higher than Hyp levels in all amniotic fluid samples; the range was 1.62–5.52 $\mu M$.

Fig. 7 shows the chromatogram of an extract of meconium-stained amniotic fluid obtained *via* an intra-uterine catheter prior to onset of labor, from a patient at 41 weeks gestation with a diagnosis of oligohydramnios and fetal compromise. Hyp and Xan peaks were pure, as indicated by purity parameters, and Hyp and

TABLE II

BASAL LEVELS OF LATE-GESTATION AMNIOTIC FLUID HYPOXANTHINE AND XANTHINE

Amniotic fluid obtained by amniocentesis at or near term, prior to onset of labor. Values are means of duplicate analyses except 4 04 $\mu M$ Xan (patient 14) which is one value Means differed from individual values by $\lesssim$5%, except 0 67 $\mu M$ Hyp (patient 2) which differed by 7 5% Purity parameters of all analyte peaks indicated that the peaks were homogenous

| Patient No | Gestational age (weeks) | Hypoxanthine ($\mu M$) | Xanthine ($\mu M$) | Indication for amniocentesis |
|---|---|---|---|---|
| 1 | 34 | 1.72 | 5 52[a] | Hypertension |
| 2 | 36 | 0.67 | 2 54[a] | Insulin-dependent diabetes |
| 3 | 36 | 0 81 | 1 62 | Discordant twins |
| 4 | 36 | 0 81 | 2.11 | Maternal cerebral aneurysm |
| 5 | 36 | 2.74 | 3.18 | Hypertension, gestational diabetes |
| 6 | 36 | 0 98 | 1 53 | Fetal hydronephrosis |
| 6 | 37 | 0 90 | 1 64 | Fetal hydronephrosis |
| 7 | 37 | 0 56 | 2 01[a] | Rh immunization |
| 8 | 37 | 0 80 | 1 63 | Possible haemophilia |
| 9 | 38 | 0 88 | 3 83[a] | Prior stillbirth |
| 10 | 38 | 1 45 | 2 36 | Gestational diabetes |
| 11 | 38 | 1.51 | 2 02 | Insulin-dependent diabetes |
| 12 | 39 | 1.32 | 1 64 | Repeat caesarian section |
| 13 | 39 | 1.45 | 2 83 | Repeat caesarian section |
| 14 | 39 | 1 85 | 4 04 | Gestational diabetes |

[a] Analysis of xanthine performed using mobile phase pH 4.5

Xan levels were 9.5 ± 0.2 and 11.2 ± 0.3 $\mu M$ (mean ± S.D. of triplicate injections), *i.e.* significantly elevated compared to the levels for 39 weeks shown in Table II.

## DISCUSSION

At term, amniotic fluid is largely fetal urine. It contains solutes which are UV-absorbing, such as uric acid, creatinine, uridine and certain amino acids and their catabolites. The complement of UV-absorbing solutes in amniotic fluid may be influenced by maternal diet, and by UV-absorbing drugs used in the treatment of the pregnant patient, which may appear in amniotic fluid unchanged or as metabolites. Thus amniotic fluid is a potentially variable biologic matrix with regard to UV-absorbing solutes, and a method of reversed-phase HPLC for analysis of amniotic fluid Hyp and Xan must achieve resolution of these analytes from a variety of major and minor UV-absorbing components. In our hands uric acid, creatinine and uridine were well resolved from Hyp and Xan on $C_{18}$ Hypersil columns under optimized mobile phase conditions, but unknown contaminant(s) co-eluted with Hyp. A column of ODS/A silica, in which free silanols are end-capped with a polar residue, achieved resolution of Hyp from the contaminant(s). Polar end-capping, which is believed to reduce clustering of the $C_{18}$ side-chains in a predominantly aqueous mobile phase and to reduce interaction of basic solutes with free silanols [12], likely also provides ion-exchange characteristics to the column. Thus mixed-mode rather than predominantly reversed-phase chromatographic separation is possible and appears to be advantageous in the resolution of unknown amniotic fluid solutes from Hyp and Xan. Keystone ODS/A is a prototype column and the nature of the polar end-capping residue has not been divulged by the manufacturer. Using the optimized chromatographic conditions, we found that Hyp and Xan in acid extracts of umbilical cord plasma were resolved as pure peaks on the Keystone ODS/A column (unpublished observations), suggesting that the mixed-mode separation this column achieves may be generally useful for resolution of the oxypurines in other biological matrices.

Because the content of unknown UV-absorbing solutes in amniotic fluid may vary from sample to sample it was necessary to confirm the purity of analyte peaks in each chromatographic analysis. Performance of xanthine oxidase peak-shift was too laborious for routine validation of peak purity. On-line computation of peak purity parameters and display of peak spectra proved to be a facile method for routine determination of peak purity.

Several earlier studies have reported Hyp levels in human amniotic fluid obtained at membrane rupture or during labor [6,13,14]. Reversed-phase HPLC of trichloroacetic acid extracts of amniotic fluid was used by one group [6,13] to measure Hyp and Xan; few methodological details were reported and no chromatograms were shown. Furthermore, in our hands, trichloroacetic acid extraction of amniotic fluid resulted in the presence of interfering artefactual peaks in the

chromatogram. In a second approach, quantitation of Hyp was performed by measurement of hydrogen peroxide formed on addition of xanthine oxidase [14]. The assumption was made that Xan levels were too low to interfere, but, as our results indicate, Xan is present in late-gestation amniotic fluid at greater basal levels than Hyp, so that results obtained by this method will be misleading.

We are not aware of any previous reports of measurement of late-gestation amniotic fluid Hyp and Xan. Our studies in fourteen patients who showed no clinical evidence of *in utero* hypoxia indicate the basal levels of these purines in human amniotic fluid in late gestation. Xan levels were higher than those of Hyp in each of the fifteen samples analyzed, although the ratio of Hyp to Xan varied from patient to patient, and was 0.2–0.6 in eleven patients and 0.8–0.9 in three patients. Under ischemic or hypoxic conditions Xan will be formed primarily from GTP as xanthine oxidase-catalyzed conversion of Hyp to Xan is inhibited, thus Xan is not an indicator of hypoxia-mediated catabolism of ATP. Variation in the ratio of amniotic fluid Hyp to Xan among the fifteen different samples suggests that formation of these metabolites is not linked. Nevertheless measurement of Xan should be a useful adjunct to measurement of Hyp in further studies investigating whether amniotic fluid Hyp concentration is related to *in utero* hypoxia.

Levels of late-gestation amniotic fluid Hyp determined in this study showed a trend towards increase with increasing gestational age, but more observations will be needed to confirm this trend. Hyp levels in the two patients with diagnosis of maternal hypertension were 2.74 and 1.72 $\mu M$. The former value from 36 weeks gestation is three-fold higher than the mean value from four other patients of the same gestational age ($0.82 \pm 0.13$ $\mu M$). The latter value is from the only patient at 34 weeks gestation, but even so, when compared with the Hyp levels in the patients at 36 weeks, is twice as high. These findings suggest that there may be a relationship between maternal hypertension and amniotic fluid Hyp levels, but more observations are needed to explore this possibility.

In the patient with oligohydramnios and strong clinical indications of fetal risk (meconium-stained amniotic fluid and fetal heart rate decelerations), amniotic fluid obtained shortly after membrane rupture contained 9.51 $\mu M$ Hyp indicating a six-fold increase over basal Hyp levels measured in three patients of 39 weeks gestation with no clinical indication of *in utero* hypoxia or fetal risk. Although the patient had reduced fluid volume the increase in Hyp concentration was substantial.

Amniotic fluid concentrations of Hyp may be affected by variations in amniotic fluid volume. Amniotic fluid volume increases during gestation to a mean of approximately 1000 ml at 34–35 weeks, after which a decrease occurs to about 800 ml at 40 weeks [15]. The volume in late gestation depends on the balance of production by the fetus, *i.e.* fetal urine and lung fluid, and removal by fetal swallowing and breathing. Small elevations in amniotic fluid Hyp could merely reflect physiologic reductions in late-gestation amniotic fluid volume. However,

for larger increases to be caused by such a mechanism, sizable fluid volume decreases would be required, which would be clinically evident by ultrasonography. In our study all amniotic fluid volumes were qualitatively normal as assessed by ultrasound at the time of amniocentesis, with the exception of the above patient with oligohydramnios.

In conclusion, we have established reliable and facile methods for sample preparation of amniotic fluid obtained by amniocentesis performed in late gestation, and for measuring the amniotic fluid content of Hyp and Xan by reversed-phase HPLC. The chromatographic method uses photodiode-array detection and a new, modified octadecylsilica column which is advantageous in resolving Hyp and Xan from other UV-absorbing amniotic fluid solutes. The chromatographic method is robust and sensitive and incorporates on-line validation of analyte peak purity. These methods were used to determine concentrations of amniotic fluid Hyp and Xan in fourteen patients from 34 to 39 weeks gestation and will provide the basis for further studies to determine whether Hyp in late-gestation amniotic fluid can serve as a biochemical marker for *in utero* hypoxia.

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