



Simultaneous high performance liquid chromatographic separation of purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids for the chemical diagnosis of inborn errors of metabolism

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Abstract

Objectives: To set up a novel simple, sensitive, and reliable ion-pairing HPLC method for the synchronous separation of several purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids for the chemical diagnosis and screening of inborn errors of metabolism (IEM).

Design and methods: The separation was set up using a Hypersil C-18, 5- μ m particle size, 250 \times 4.6 mm column, and a step gradient using two buffers and tetrabutylammonium hydroxide as the pairing reagent. A highly sensitive diode array UV detector was set up at a wavelength between 200 and 300 nm that revealed purines and pyrimidines at 260 nm and other compounds at 206 nm.

Results: Compounds were determined in the plasma of 15 healthy adults, in the urine of 50 healthy subjects (1–3 years, 4–6 years, 8–10 years, 12–18 years, 25–35 years), and in 10 non-pathological amniotic fluid samples. To assess the validity of the chemical diagnosis of IEM, plasma and urine samples were analyzed in patients affected by Canavan disease ($n = 10$; mean age 4.6 ± 2.3). Low plasma levels of *N*-acetylaspartate ($16.96 \pm 19.57 \mu\text{mol/L}$ plasma; not detectable in healthy adults) and dramatically high urinary *N*-acetylaspartate concentrations ($1872.03 \pm 631.86 \mu\text{mol/mmol creatinine}$; 450 times higher than that which was observed in age-matched controls) were recorded. Neither *N*-acetylglutamate nor *N*-acetylaspartylglutamate could be detected in the plasma or urine of controls or patients with Canavan disease.

Conclusions: The results demonstrate the suitability of the present ion-pairing HPLC separation with UV detection of cytosine, cytidine, creatinine, uracil, uridine, β -pseudouridine, adenine, 3-methyladenine, hypoxanthine, xanthine, xanthosine, inosine, guanosine, ascorbic acid, thymine, thymidine, uric acid, 1-methyluric acid, orotic acid, *N*-acetylaspartate, *N*-acetylglutamate, *N*-acetylaspartylglutamate, malonic acid, methylmalonic acid, GSH, and GSSG as a reliable method for the prenatal and neonatal chemical diagnosis and screening of IEM using biological fluids.

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Keywords: Inborn errors of metabolism; Purines; Pyrimidines; *N*-acetylated amino acids; Dicarboxylic acids; HPLC; Chemical diagnosis; Prenatal and neonatal screening; Biological fluids; Canavan disease

Introduction

Detection of purine and pyrimidine derivatives of *N*-acetylated amino acids and dicarboxylic acids in biological fluids is critically important in the chemical diagnosis of

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several inborn errors of metabolism (IEM). Table 1 is a short list of some, often fatal, IEM as well as some compounds which show significant variations with respect to normal values. Therefore, the chemical analysis of biological fluids for the screening of IEM is crucial to achieve prenatal or neonatal diagnosis, as well as to monitor disease progression and evaluate the effectiveness of therapeutic interventions. For these reasons, analytical methods used to evaluate the compounds of interest must be characterized by high sensitivity, reproducibility, and specificity. Furthermore, in order for these analytical methods to have a wide range of application in clinical biochemistry laboratories, they need to be easy to perform and relatively inexpensive. In addition, in order to ensure reliable analytical data, any method considered must reduce to a minimum sample manipulation.

The compounds listed below, the concentrations of which are altered in various genetic metabolic disorders and in several acute and chronic pathological states, are all very different in terms of their chemical nature. *N*-acetylaspartate (NAA), *N*-acetylglutamate (NAG), *N*-acetylaspartylgluta-

mate (NAAG), and dicarboxylic acids are ionizable compounds, with two (or three in the case of NAAG) negative charges at neutral pH; purine and pyrimidine nucleosides are polar, non-ionizable substances; free purines and pyrimidines (and most of their derivatives) are low-polar or non-polar compounds. The degree of polarity of nucleosides, as well as free purines and pyrimidines, varies significantly depending on their base type (purines are more electronegative than pyrimidines) and the chemical nature of additional groups, which are eventually added to the heterocyclic ring(s) (electron attractors versus electron repulsors). Several analytical methods have been described for the determination of purines, pyrimidines [1–10], NAA, NAAG [11–15], and dicarboxylic acids [16–20] in biological fluids, with the ultimate goal of screening for IEM. However, none of them have attempted to separate and measure compounds of different chemical classes in a single analysis.

The majority of the methods in the literature for the analysis of NAA [11–15] and dicarboxylic acids [16–20] are based on the use of gas chromatography/mass spectrometry (GC/MS), while several HPLC assays have been described for the determination of purines and pyrimidines [1–5,9,10]. Although GC/MS may be preferable to HPLC in some cases due to the efficiency of the column and the selectivity of the detector, it is certainly not the method of choice for the separation of polar compounds. In fact, to allow its application in separating ionizable and polar compounds such as *N*-acetylated amino acids, dicarboxylic acids, and nucleosides, complex sample processing which often results in modest sample recovery is required [19]. For example, samples of all origins require trimethylsilyl derivatization and in the case of urine, urease pretreatment is also necessary; stable isotopic dilution is also required for compounds in low concentrations [12,14,19].

In this study, we report a highly sensitive, reproducible, and simple ion-pairing HPLC method with UV detection, characterized by the absence of sample manipulation and for the simultaneous separation and quantification of representative compounds of purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids, as well as of creatinine, GSH, GSSG, and ascorbic acid in biological fluids. To validate the suitability of the present HPLC method for the prenatal and neonatal chemical diagnosis of IEM, we measured different metabolites in plasma and urine samples from both healthy subjects as well as patients with Canavan disease.

Methods

Chemicals

Tetrabutylammonium hydroxide was used as the pairing reagent for the HPLC separation of metabolites and was obtained as a 55% water solution from Nova Chimica (Milan, Italy). The HPLC-grade methanol was supplied by Carlo Erba Reagenti (Milan, Italy). The Ultrapure HPLC

Table 1

Main inborn errors of metabolism (IEM) diagnosable through the analysis of purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids in biological fluids

IEM	Detectable metabolites in biological fluids
1. Hypoxanthine phosphoribosyl transferase deficiency (Lesch–Nyhan syndrome)	Hypoxanthine, xanthine, uric acid, inosine, guanosine, adenine
2. Phosphoribosylpyrophosphate synthetase deficiency	Hypoxanthine, xanthine, uric acid
3. Purine nucleoside phosphorylase deficiency	Uric acid, inosine, guanosine
4. Xanthine dehydrogenase deficiency	Hypoxanthine, xanthine, uric acid
5. Adenine phosphoribosyl transferase deficiency	Adenine
6. Uridine monophosphate synthetase deficiency	Orotic acid
7. Dihydropyrimidine dehydrogenase deficiency	Uracil, thymine
8. Mitochondrial neuro-gastro-intestinal encephalopathy from thymidine kinase deficiency	Thymine, thymidine, uracil
9. Mitochondrial myopathy and sideroblastic anemia from defective β -pseudouridine synthase	β -Pseudouridine
10. Dyskeratosis congenita	β -Pseudouridine
11. <i>N</i> -acetylasparto-acylase deficiency (Canavan disease)	<i>N</i> -acetylaspartate, <i>N</i> -acetylglutamate?, <i>N</i> -acetylaspartylglutamate?, <i>N</i> -acetylaspartylglutamate
12. Neurometabolic disorder of still unknown origin and resembling the clinical features of the Pelizaeus–Merzbacher syndrome	
13. Methylmalonic aciduria	Methylmalonic acid
14. Malonic aciduria	Malonic acid

standards were provided either by Sigma (St. Louis, Mo, USA) or by ICN-MP Biochemicals (Irvine, California, USA) at the highest purity available. Other reagents were purchased from commercial sources.

Patient selection and biological fluid sampling and preparation

Fifteen plasma samples from healthy volunteers from our laboratories (27–50 years, mean age 42.3 ± 18.6) were

obtained by withdrawing supernatants of centrifuged, heparin-treated blood ($1860 \times g$ for 10 min at 4°C). Urinary samples from fifty subjects with no metabolic disorders were assayed by the Clinical Biochemistry Laboratory of the “Policlinico A. Gemelli” for routine analyses. They were divided into five groups depending on the age of the donor (1–3 years, 4–6 years, 8–10 years, 12–18 years, and 25–35 years) and were used as control groups. Ten cell-free samples of amniotic fluid, assayed by the Clinical Biochemistry Laboratory of the “Policlinico A. Gemelli” for routine

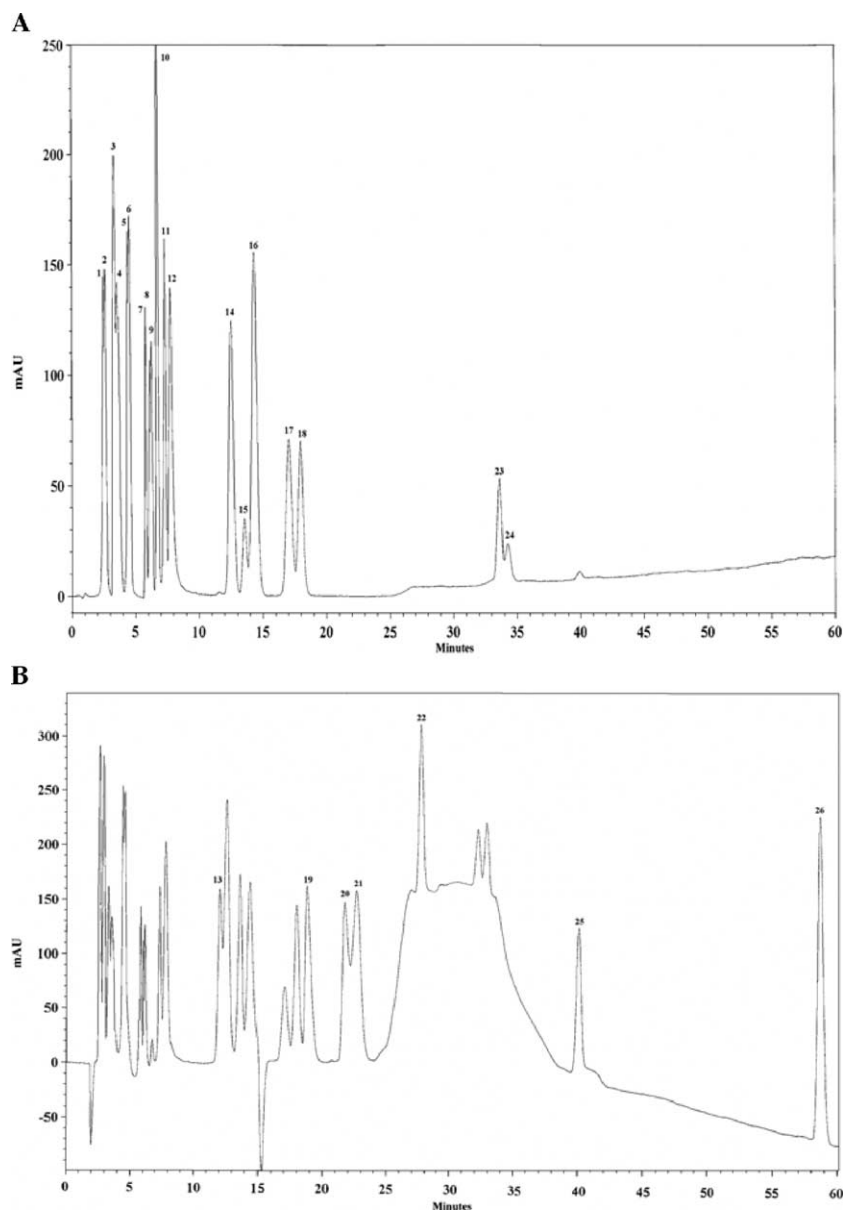


Fig. 1. Separation of a standard mixture containing 5–200 μM cytosine, cytidine, creatinine, uracil, uridine, β -pseudouridine, adenine, 3-methyladenine, hypoxanthine, xanthine, xanthosine, inosine, guanosine, ascorbic acid, thymine, thymidine, uric acid, 1-methyluric acid, orotic acid (Panel A, 260 nm wavelength), NAA, NAG, NAAG, malonic acid, methylmalonic acid, GSH, and GSSG (Panel B, 206 nm wavelength). Sample processing and chromatographic conditions are described in detail under the Methods section. (1) cytosine; (2) creatinine; (3) uracil; (4) β -pseudouridine; (5) cytidine; (6) hypoxanthine; (7) thymine; (8) 3-methyladenine; (9) xanthine; (10) ascorbic acid; (11) uridine; (12) adenine; (13) GSH; (14) inosine; (15) uric acid; (16) guanosine; (17) thymidine; (18) orotic acid; (19) malonic acid; (20) methylmalonic acid; (21) *N*-acetylaspartate; (22) *N*-acetylglutamate; (23) 1-methyluric acid; (24) xanthosine; (25) GSSG; and (26) *N*-acetylaspartylglutamate.

analyses, were obtained from women between the 12th and the 16th week of pregnancy. Ten urinary and plasma samples from patients with Canavan disease (0.5–6 years, mean age 4.6 ± 2.3), hospitalized by the Robert Wood Johnson Medical School for Gene Therapy for Canavan Disease, University of Medicine and Dentistry of New Jersey, USA, were obtained as described above. Samples of urine, plasma, and amniotic fluid from controls were diluted with bi-distilled water 25, 5, and 3 times, respectively. Urinary and plasma samples from patients with Canavan disease were diluted with bi-distilled water 400 and 10 times, respectively. Aliquots of all samples were transferred in an Eppendorff tube equipped with a filtering membrane of 3 kDa cut-off (Nanosep® Centrifugal Devices, Pall Gelman Laboratory, Ann Harbor, MI, USA) and centrifuged at $10,500 \times g$ for 30 min at 4°C. The deproteinized ultrafiltrate fluid was directly injected onto the HPLC column.

HPLC equipment and conditions for the separation of purines, pyrimidines, N-acetylated amino acids, and dicarboxylic acids

Freshly prepared standard mixtures, with known concentrations and aliquots of each biological fluid sample (200 µL), were assayed by ion-pairing HPLC for the separation of cytosine, cytidine, creatinine, uracil, uridine, β-pseudouridine, adenine, 3-methyladenine, hypoxanthine, xanthine, xanthosine, inosine, guanosine, ascorbic acid, thymine, thymidine, uric acid, 1-methyluric acid, orotic acid, NAA, NAG, NAAG, malonic acid, and methylmalonic acid. Since reduced (GSH) and oxidized glutathione (GSSG) could also be separated under the chromatographic conditions used, they were added to the standard mixtures.

The HPLC apparatus consisted of a SpectraSystem P2000 pump and a highly sensitive UV6000LP diode array detector (ThermoElectron Italia, Rodano, Milan, Italy), equipped with a 5-cm light-path flow cell and was set up with a wavelength between 200 and 300 nm. Data were acquired and analyzed by a PC using the ChromQuest® software package provided by the HPLC manufacturer. Separation of the various compounds was carried out using a Hypersil 250 × 4.6 mm, 5 µm particle-size column, which was provided with its own guard column (ThermoElectron Italia, Rodano, Milan, Italy). A step gradient from buffer A (12 mM tetrabutylammonium hydroxide, 10 mM KH₂PO₄, 0.125% methanol, pH 7.00) to buffer B (2.8 mM tetrabutylammonium hydroxide, 100 mM KH₂PO₄, 30% methanol, pH 5.50) was formed as follows: 20 min at 100% buffer A; 8 min at up to 80% buffer A; 10 min at up to 70% buffer A; 12 min at up to 55% buffer A; 11 min at up to 40% buffer A; 9 min at up to 15% buffer A; 10 min at up to 0% buffer A; hold for column washing 0% buffer A for an additional 20 min. A flow rate of 1.2 mL/min and a column temperature of 10°C

were maintained constant throughout the analysis. Species identification, using deproteinized samples of biological fluids, was determined by matching retention times and absorption spectra to freshly prepared ultrapure standards. If needed, co-chromatograms were performed by adding known standards to the biological samples. The concentration of the different compounds were calculated from the standard run data at the wavelengths corresponding to the peak absorption of each substance (206 nm wavelength: NAA, NAG, NAAG, GSH, GSSG, malonic acid, and methylmalonic acid; 260 nm wavelength: cytosine, cytidine, uracil, uridine, β-pseudouridine, adenine, 3-methyladenine, hypoxanthine, xanthine, xanthosine, inosine, guanosine, ascorbic acid, thymine, thymidine, uric acid, 1-methyluric acid, and orotic acid; 234 nm wavelength: creatinine).

Statistics

Statistical differences between controls and Canavan patients were tested by the one-way analysis of variance

Table 2

Lower limit of detection, linearity and limit of quantification of the new ion-pairing HPLC method for the detection of purines, pyrimidines, N-acetylated amino acids, and dicarboxylic acids in biological fluids

	LLOD ^a (µM)	500 × LLOD (µM)	Correlation coefficient ^b	LOQ ^c (µM)
Cytosine	0.05	25	0.998	0.08
Cytidine	0.1	50	0.996	0.15
Creatinine	0.05	25	0.986	0.08
Uracil	0.05	25	0.997	0.08
Uridine	0.05	25	0.999	0.08
β-Pseudouridine	0.05	25	0.999	0.08
Adenine	0.05	25	0.995	0.08
3-Methyladenine	0.05	25	0.993	0.08
Hypoxanthine	0.05	25	0.998	0.04
Xanthine	0.05	25	0.998	0.08
Xanthosine	0.05	25	0.994	0.08
Inosine	0.05	25	0.996	0.08
Guanosine	0.05	25	0.994	0.08
Ascorbic acid	0.05	25	0.999	0.04
Thymine	0.05	25	0.993	0.04
Thymidine	0.1	50	0.994	0.15
Uric acid	0.15	75	0.999	0.20
1-Methyluric acid	0.15	75	0.999	0.20
Orotic acid	0.15	75	0.997	0.20
NAA	0.35	175	0.999	0.40
NAG	0.35	175	0.999	0.40
NAAG	0.18	90	0.999	0.25
Malonic acid	2	1000	0.991	3
Methylmalonic acid	2	1000	0.993	3
GSH	0.15	75	0.996	0.20
GSSG	0.05	25	0.997	0.08

^a LLOD = Lower limit of detection, evaluated with a signal to noise ratio >3.

^b Linearity was determined by assaying standard mixtures with the following concentrations: LLOD, 10 × LLOD, 50 × LLOD, 100 × LLOD, and 500 × LLOD.

^c LOQ = limit of quantification, evaluated with a signal to noise ratio >5.

(ANOVA) and the Fisher's PLSD post hoc test. Differences were regarded as statistically significant at $P < 0.05$.

Results

HPLC separation of a standard mixture

In Fig. 1, a representative chromatogram of a standard mixture containing 5–200 μM cytosine, cytidine, creatinine, uracil, uridine, β -pseudouridine, adenine, 3-methyladenine, hypoxanthine, xanthine, xanthosine, inosine, guanosine, ascorbic acid, thymine, thymidine, uric acid, 1-methyluric acid, orotic acid (A), NAA, NAG, NAAG, malonic acid, methylmalonic acid, GSH, and GSSG (B) is reported. Cytosine and creatinine, uracil and β -pseudouridine, cytidine and hypoxanthine, and 3-methyladenine and xanthine were not fully resolved (A). However, with the aid of spectral differences, reproducible quantization of these compounds may be obtained. Peaks with a k' of 11.78 for NAA, 14.33 for NAG, and 31.67 for NAAG (where $k' = \frac{V - V_0}{V_0}$, with V corresponding to the elution volume and V_0 to the void volume) were obtained under these chromatographic conditions.

Table 3

Reproducibility of the new ion-pairing HPLC method for the detection of purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids in biological fluids

	Coefficients of variation of retention times (%)	Coefficients of variation of peak areas (%)
Cytosine	0.11 \pm 0.03	0.56 \pm 0.05
Cytidine	0.22 \pm 0.07	1.65 \pm 0.12
Creatinine	0.11 \pm 0.04	0.68 \pm 0.10
Uracil	0.14 \pm 0.03	0.67 \pm 0.06
Uridine	0.25 \pm 0.02	0.88 \pm 0.09
β -Pseudouridine	0.13 \pm 0.02	0.49 \pm 0.03
Adenine	0.18 \pm 0.02	1.10 \pm 0.10
3-Methyladenine	0.23 \pm 0.08	1.24 \pm 0.13
Hypoxanthine	0.10 \pm 0.02	0.66 \pm 0.09
Xanthine	0.27 \pm 0.03	1.21 \pm 0.11
Xanthosine	0.34 \pm 0.09	1.05 \pm 0.05
Inosine	0.30 \pm 0.01	1.32 \pm 0.13
Guanosine	0.36 \pm 0.05	1.36 \pm 0.15
Ascorbic acid	0.17 \pm 0.02	0.71 \pm 0.08
Thymine	0.21 \pm 0.04	1.11 \pm 0.09
Thymidine	0.27 \pm 0.03	1.53 \pm 0.03
Uric acid	0.09 \pm 0.01	1.30 \pm 0.08
1-Methyluric acid	0.13 \pm 0.01	0.98 \pm 0.05
Orotic acid	0.51 \pm 0.04	1.28 \pm 0.13
NAA	0.28 \pm 0.02	0.74 \pm 0.07
NAG	0.32 \pm 0.03	0.93 \pm 0.04
NAAG	0.17 \pm 0.03	1.01 \pm 0.11
Malonic acid	0.25 \pm 0.03	1.03 \pm 0.02
Methylmalonic acid	0.20 \pm 0.02	1.34 \pm 0.12
GSH	0.28 \pm 0.04	1.22 \pm 0.14
GSSG	0.12 \pm 0.03	0.87 \pm 0.09

Each point is the mean \pm SD of five different standard mixtures assayed for five consecutive days.

Table 4

Recovery of the new ion-pairing HPLC method for the detection of purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids in biological fluids

	$\mu\text{mol/L}$ plasma	$\mu\text{mol/L}$ urine
Cytosine	4.98 \pm 0.36	4.78 \pm 0.31
Cytidine	4.91 \pm 0.28	5.12 \pm 0.38
Creatinine	5.21 \pm 0.52	5.16 \pm 0.28
Uracil	5.12 \pm 0.35	5.23 \pm 0.52
Uridine	4.77 \pm 0.23	5.32 \pm 0.29
β -Pseudouridine	5.12 \pm 0.47	4.96 \pm 0.36
Adenine	4.95 \pm 0.15	5.01 \pm 0.12
3-Methyladenine	4.88 \pm 0.36	4.90 \pm 0.37
Hypoxanthine	4.76 \pm 0.54	4.93 \pm 0.41
Xanthine	4.91 \pm 0.17	5.17 \pm 0.44
Xanthosine	5.08 \pm 0.30	5.32 \pm 0.24
Inosine	4.91 \pm 0.31	4.88 \pm 0.51
Guanosine	4.89 \pm 0.20	4.84 \pm 0.33
Ascorbic acid	4.94 \pm 0.26	5.12 \pm 0.58
Thymine	4.97 \pm 0.18	5.35 \pm 0.22
Thymidine	4.88 \pm 0.16	5.13 \pm 0.27
Uric acid	5.02 \pm 0.29	5.11 \pm 0.49
1-Methyluric acid	4.87 \pm 0.24	4.92 \pm 0.32
Orotic acid	5.06 \pm 0.29	5.08 \pm 0.66
NAA	4.93 \pm 0.18	4.97 \pm 0.32
NAG	5.17 \pm 0.22	4.75 \pm 0.48
NAAG	4.89 \pm 0.12	4.96 \pm 0.30
Malonic acid	4.98 \pm 0.31	4.86 \pm 0.36
Methylmalonic acid	4.99 \pm 0.28	4.97 \pm 0.21
GSH	4.83 \pm 0.31	5.16 \pm 0.49
GSSG	5.11 \pm 0.35	5.02 \pm 0.54

Each value is the mean \pm SD of five plasma and urinary samples spiked with standard mixtures with known concentrations. Sample processing and chromatographic conditions are fully described in the Methods section.

To validate the present HPLC separation, lower limit of detection (LLOD), linearity, limit of quantification (LOQ), and reproducibility were evaluated. Table 1 summarizes values of LLOD (evaluated with a signal to noise ratio >3), linearity, and LOQ (calculated with a signal to noise ratio >5) of the various compounds considered. Minimal values of LLOD were observed for cytosine, creatinine, uracil, uridine, β -pseudouridine, adenine, 3-methyladenine, hypoxanthine, xanthine, xanthosine, inosine, guanosine, ascorbic acid, thymine, and GSSG (50 nM, corresponding to 10 pmol/200 μL injected). In contrast, maximal values of LLOD were recorded for malonic and methylmalonic acid (4 μM , corresponding to 0.8 nmol/200 μL injected). In the concentration range between LLOD and $500 \times$ LLOD, high linearity was observed for all of the compounds separated as indicated by the values of the correlation coefficients (three intermediate concentrations for each analyte were considered between LLOD and $500 \times$ LLOD). The high reproducibility of this new chromatographic separation was confirmed by the constancy of retention times and peak areas determined for five standard mixtures prepared on five consecutive days. The data reported in Table 2 indicate that orotic acid had the highest coefficient of variation of retention time and cytidine had the highest coefficient of variation of peak area.

Table 5

Purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids in plasma from healthy adults determined according to the new ion-pairing HPLC method

	μmol/L plasma
Cytidine	0.25 ± 0.19
Creatinine	74.12 ± 10.91
Uracil	2.10 ± 1.02
Uridine	3.12 ± 1.31
β-Pseudouridine	3.18 ± 0.99
Hypoxanthine	4.87 ± 0.36
Xanthine	1.27 ± 0.78
Inosine	0.20 ± 0.07
Ascorbic acid	62.57 ± 4.71
Uric acid	271.95 ± 43.13
Thymidine	0.21 ± 0.13
Orotic acid	0.89 ± 0.63
GSH	37.03 ± 4.76
GSSG	1.69 ± 0.38

Each value is the mean ± SD of fifteen plasma samples. Sample preparation and chromatographic conditions are fully described in the Methods section.

Evaluation of the HPLC separation of purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids in biological fluids

To assess the suitability of the described sample processing (deproteinization by filtration through membranes with a 3-kDa cut-off) and of this new HPLC method for the quantification of cytosine, cytidine, creatinine, uracil, uridine, β-pseudouridine, adenine, 3-methyladenine, hypoxanthine, xanthine, xanthosine, inosine, guanosine, ascorbic acid, thymine, thymidine, uric acid, 1-methyluric acid, orotic acid, NAA, NAG, NAAG, malonic acid, methylmalonic acid, GSH, and GSSG, we conducted a preliminary evaluation of the efficiency of the compound recovery. For this purpose, plasma and urine were spiked with a mixture containing known concentrations of each compound and these enriched

samples were processed and analyzed as described above. Data reported in Table 3 demonstrate the high efficiency of metabolite recovery with values oscillating from a minimum of 95.2% (3-methyladenine) to a maximum of 104.2% (creatinine).

To appraise the reliability of this new HPLC analysis for the simultaneous separation of purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids in biological fluids, paying particular attention to its potential use as a reliable test for prenatal and neonatal screening of IEM, we applied the previously described sample processing and chromatographic conditions to determine concentrations of the aforementioned compounds in plasma and urinary samples from control healthy subjects of different ages, as well as in amniotic fluid specimens. As reported in Table 4, in the plasma from control subjects cytosine, adenine, 3-methyladenine, xanthosine, guanosine, 1-methyluric acid, *N*-acetylated amino acids (NAA, NAG, and NAAG), and dicarboxylic acids (malonic and methylmalonic acids) were below the LLOD of the method. It is worth underlining that both GSH and GSSG could be measured in all samples. As shown in Table 5, urinary samples of healthy subjects were divided in five different subgroups depending on the age of the donor. In all samples, cytosine, uridine, adenine, xanthosine, guanosine, thymine, ascorbic acid, NAG, NAAG, malonic acid, GSH, and GSSG were below the LLOD of the method. Interestingly, with increasing mean age of donor, concentrations of β-pseudouridine, 3-methyladenine, hypoxanthine, xanthine, uric acid, NAA, and methylmalonic acid decreased significantly while thymidine and 1-methyluric acid increased progressively. Table 6 reports values of metabolites determined in ten samples of amniotic fluids withdrawn between the 12th and the 16th week of pregnancy. Cytosine, cytidine, uridine, adenine, 3-methyladenine, thymine, inosine, xanthosine, guanosine, *N*-acetylated amino acids (NAA, NAG, and NAAG), dicarbox-

Table 6

Purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids in urine from control healthy subjects determined according to the new ion-pairing HPLC method

	Aged 1–3 years (μmol/mmol creatinine)	Aged 4–6 years (μmol/mmol creatinine)	Aged 8–10 years (μmol/mmol creatinine)	Aged 12–18 years (μmol/mmol creatinine)	Aged 25–35 years (μmol/mmol creatinine)
Cytidine	1.97 ± 1.28	0.93 ± 0.72	1.50 ± 1.34	1.12 ± 0.69	0.33 ± 0.17
Uracil	14.10 ± 2.88	7.77 ± 3.43	10.13 ± 5.43	6.81 ± 5.09	12.29 ± 5.78
β-Pseudouridine	90.78 ± 32.15	57.89 ± 28.18	50.02 ± 12.77	42.17 ± 9.60	36.01 ± 9.52
3-Methyladenine	2.93 ± 0.98	2.64 ± 2.29	2.53 ± 1.65	1.48 ± 0.17	0.86 ± 0.29
Hypoxanthine	17.15 ± 5.85	10.66 ± 0.62	5.78 ± 3.99	4.82 ± 2.20	2.30 ± 2.48
Xanthine	25.53 ± 7.97	19.30 ± 7.05	13.31 ± 7.84	8.71 ± 2.61	4.89 ± 1.50
Inosine	1.22 ± 1.41	0.32 ± 0.38	0.71 ± 0.89	0.28 ± 0.33	1.04 ± 0.56
Uric acid	821.68 ± 255.76	668.88 ± 336.58	524.75 ± 249.57	337.84 ± 29.75	209.55 ± 104.52
1-Methyluric acid	10.58 ± 3.37	12.94 ± 5.38	13.95 ± 10.65	25.20 ± 13.50	32.56 ± 17.81
Thymidine	1.22 ± 0.94	4.55 ± 2.70	2.46 ± 2.03	1.38 ± 1.02	6.87 ± 4.86
Orotic acid	0.46 ± 0.45	0.38 ± 0.11	0.23 ± 0.13	0.16 ± 0.12	N.D.
NAA	3.41 ± 1.59	4.80 ± 3.04	1.65 ± 1.50	0.83 ± 0.83	0.40 ± 0.43
Methylmalonic acid	9.24 ± 4.86	10.25 ± 5.29	0.64 ± 0.39	0.17 ± 0.11	2.51 ± 1.01

Controls were divided into five subgroups of different ages, of ten subjects each. Values are the mean ± SD of ten different urinary samples. Sample processing and chromatographic conditions are fully described in the Methods section.

ylic acids (malonic and methylmalonic acids), GSH, and GSSG were below the LLOD of the method. In one sample, ascorbic acid was undetectable, while 1-methyluric acid was recorded in 3/10 samples only. The presence of ascorbic acid in most of the samples was probably due to the 1 g/day administration of vitamin C during the pregnancy, as confirmed by the caring gynecologists. It is worth emphasizing that donors of amniotic fluids delivered neonates with no IEM. Bearing in mind the limited number of specimens, concentrations of the different metabolites determined in these amniotic fluid samples can therefore be considered as normal control values.

To evaluate the suitability of the present method for the chemical diagnosis of IEM, we assayed plasma and urinary samples from a cohort of patients suffering from Canavan disease. A representative chromatogram of a deproteinized

plasma specimen from a Canavan patient (Fig. 2, A) was compared with that of an age-matched control (Fig. 2, B). The 206-nm wavelength trace of the Canavan patient's plasma clearly shows a peak with k' identical to that of standard NAA (Fig. 1, B). A co-chromatogram of the same Canavan patient's plasma sample spiked with a known concentration of standard NAA confirmed the assignment of the peak to NAA (data not shown). Table 7 summarizes values of the various metabolites determined in the plasma of Canavan patients. Mean NAA concentration of $16.96 \pm 19.57 \mu\text{mol/L}$ plasma) was found. An anomalous value of $1246.71 \mu\text{mol/L}$ plasma was recorded in one sample (run in quadruplicate) and was omitted from the calculation of the mean NAA value. Interestingly, we found a mean value of $16.70 \pm 3.72 \mu\text{mol/L}$ plasma for β -pseudouridine, a value about 5.3 times higher than the value recorded in

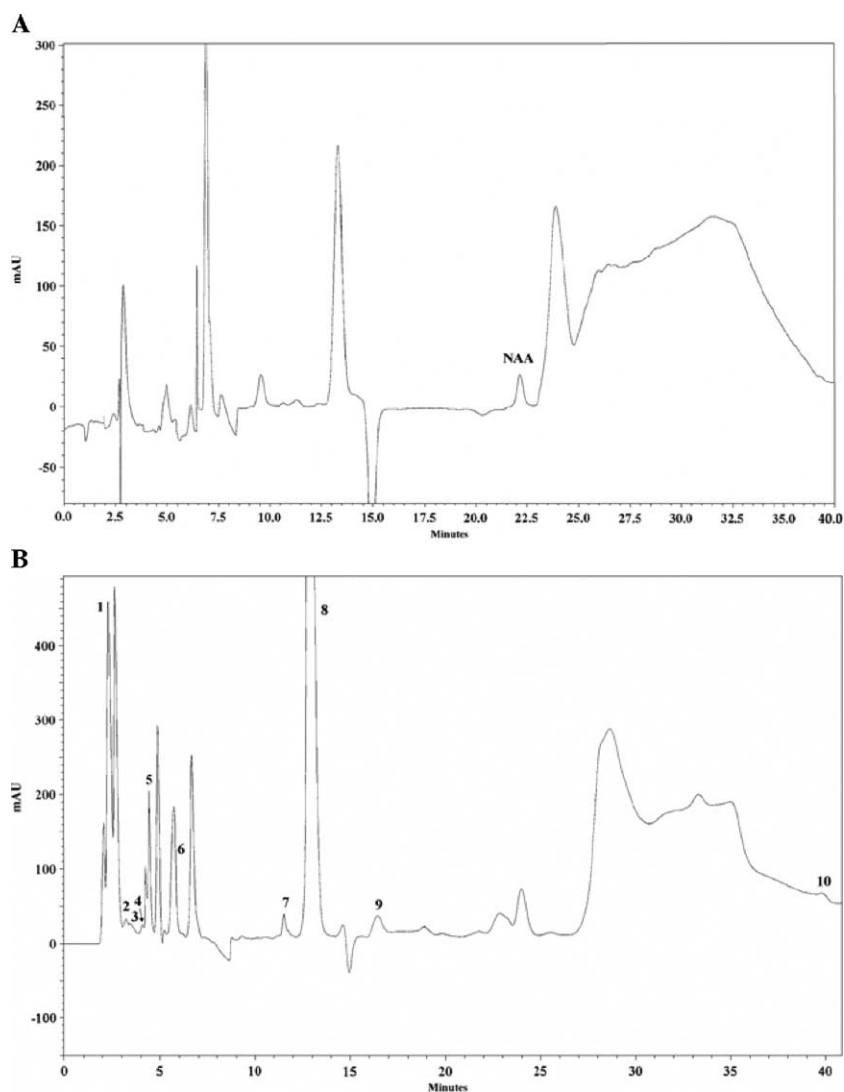


Fig. 2. Representative 206-nm wavelength chromatographic trace of a deproteinized plasma sample (200 μL injected) from a Canavan patient (A) and an adult healthy subject (B). No NAA was detected in the control sample (sample diluted 5-fold). A significant NAA peak in the Canavan patient plasma was observed (sample diluted 10-fold). Neither NAG nor NAAG were detectable in samples from both the Canavan patient and the control. Sample processing and chromatographic conditions are described in detail under the Methods section. (1) creatinine; (2) uracil; (3) β -pseudouridine; (4) cytidine; (5) hypoxanthine; (6) xanthine; (7) GSH; (8) uric acid; (9) thymidine; (10) GSSG.

Table 7

Purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids in non-pathological amniotic fluid samples determined according to the new ion-pairing HPLC method

	μmol/L amniotic fluid
Creatinine	69.65 ± 9.88
Uracil	0.87 ± 0.69
β-Pseudouridine	1.29 ± 1.07
Hypoxanthine	0.086 ± 0.044
Xanthine	0.48 ± 0.24
Ascorbic acid	20.04 ± 12.21
Uric acid	384.18 ± 203.31
1-Methyluric acid	0.51 ± 0.044
Thymidine	1.52 ± 1.21
Orotic acid	0.27 ± 0.25

Values are the mean ± SD of ten different amniotic fluid samples, withdrawn between the 12th and the 16th week of pregnancy. Sample processing and chromatographic conditions are fully described in the Methods section.

the plasma of adult healthy subjects ($P < 0.01$). Neither NAG nor NAAG were recorded in any of these plasma samples.

Comparison of representative chromatographic traces of a deproteinized urinary sample from a Canavan patient (Fig. 3, A) and an age-matched control (Fig. 3, B) demonstrates the presence of a large peak with a k' identical to that of true NAA (Fig. 1, B). Also, in this case, the co-chromatogram of the same Canavan urinary sample spiked with a known concentration of standard NAA confirmed the assignment of the peak to NAA (data not shown). It is worth recalling that all urine samples from Canavan patients (including the one whose trace is reported in Fig. 3, A) were diluted 400 times before injection, while those of controls were diluted 25 times. The values of the different compounds detected in the urinary samples from the cohort of Canavan patients are summarized in Table 8. Similar to the results for plasma, significant differences with respect to values recorded in age-matched controls were evident only for NAA (1872.03 ± 631.86 μmol/mmol creatinine; $P < 0.001$) and β-pseudouridine (319.27 ± 198.36 μmol/mmol creatinine; $P < 0.01$). Neither NAG nor NAAG were recorded in any of these urinary samples even when they were subjected to a lower dilution (1:100) prior to the HPLC analysis (Table 9).

Discussion

Data reported in the present study demonstrate the validity of a new ion-pairing HPLC analysis for the separation of purines, pyrimidines, *N*-acetylated amino acids, dicarboxylic acids, GSH, and GSSG in biological fluids, which is particularly suitable for prenatal and neonatal chemical diagnosis of IEM associated with the aforementioned compounds.

With respect to previously reported methods, this HPLC assay has some unique advantages: (a) none of the other methods presently available allow the separation and

quantification of all of the present compounds considered with a single analysis; (b) the sample preparation applied here requires manipulation limited only to the removal of proteins by ultrafiltration through a membrane with a 3-kDa cut-off prior to the injection into the HPLC column; (c) the sensitivity of this assay is such that a reduced sample volume of 10 μL and 100 μL is required for urine and plasma, respectively; (d) this method has the potential to separate (for the first time to the best of our knowledge) NAA, NAG, and NAAG; (e) this method is applicable to any biological fluid with the same characteristics of efficiency, reproducibility, and sensitivity; (f) the relatively low cost of both the instruments and supplies used, the ease of execution, the robustness of HPLC devices and columns; (g) since all the compounds associated with the 14 IEM listed in Table 1 are eluted within 35 min, and since no NAAG (retention time ≈ 58 min) was detected in both normal and pathological body fluids, the total time between two consecutive injections (analysis + column washing + column equilibration) can be shortened to about 60 min with only a three-step gradient and no loss of information. Therefore, the assay is suitable for the analysis of a large number of samples for prenatal and neonatal screening of at least 14 IEM.

The HPLC separation presented here was possible thanks to the use of a C-18 reversed-phase HPLC column coupled with an ion-pairing reagent (tetrabutylammonium hydroxide), which was added to the mobile phase; we have previously applied this method with success in the separation of compounds with variable polarity in complex biological mixtures [21–26].

As summarized in Tables 4 and 5, by applying the present HPLC analysis, we found the values for purines, pyrimidines, nucleosides, ascorbic acid, GSH, and GSSG in the plasma and urine of controls comparable to those reported in previous studies [1–20,27–30]. Similarly, most of the age-related differences observed between the different subgroups for some of the compounds considered (β-pseudouridine, 3-methyladenine, hypoxanthine, xanthine, uric acid, NAA, methylmalonic acid, thymidine, and 1-methyluric acid) were comparable to data reported in the literature [10]. The application of this method, for the analysis of pathological samples from biological fluids, was tested using plasma and urinary samples from patients suffering from Canavan disease. In this pathological condition, large quantities of NAA accumulate in the brain of infants due to the synthesis of inactive forms of the NAA-degrading enzyme, *N*-acetylasparto acylase (ASPA). Plasma and urinary NAA concentrations increase significantly and primary clinical diagnosis is based on NAA determination in biological fluids [12,14,31]. Since it is visible in the far-UV region only, NAA is mainly determined by GC/MS. This analytical method requires sample derivatization and, in the case of urine, urease pretreatment. As shown in Figs. 2 and 3, as a consequence of the high concentration of NAA in the urine and plasma specimens from Canavan patients, we were forced to dilute

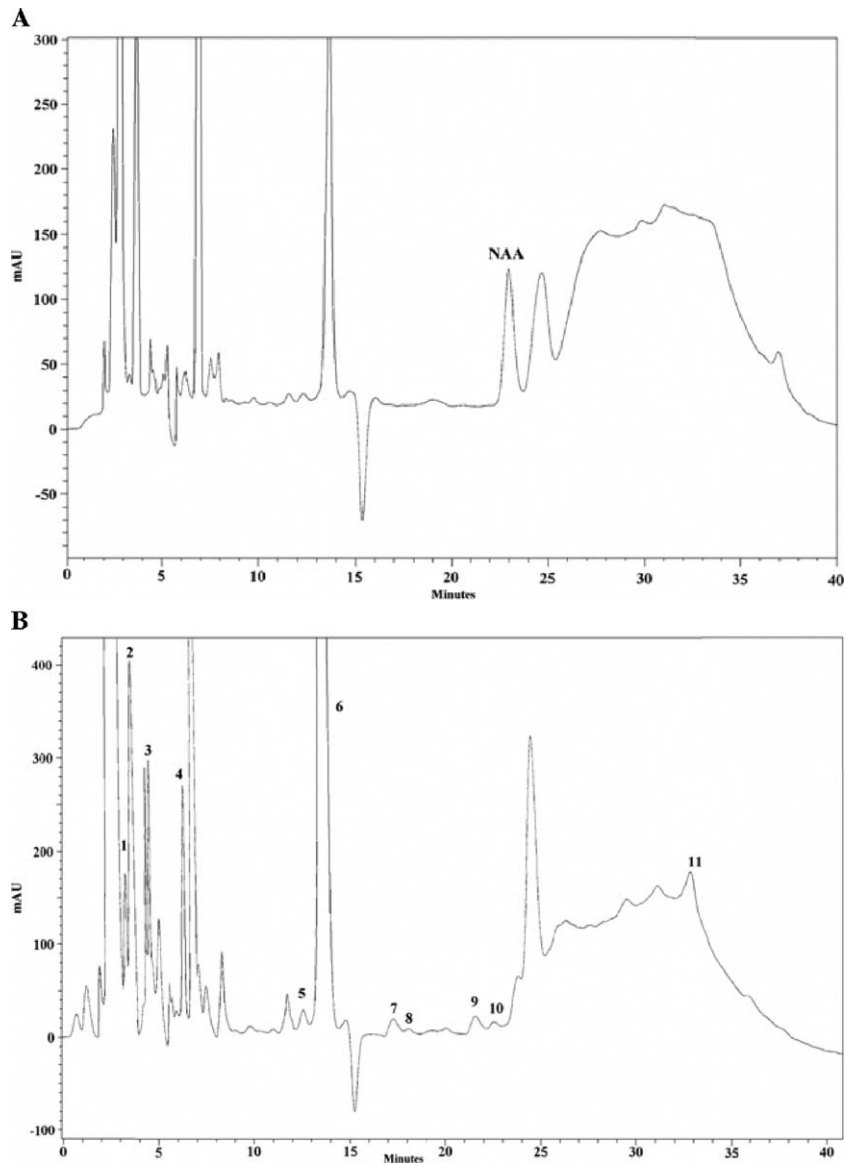


Fig. 3. Representative 206-nm wavelength chromatographic trace of a deproteinized urinary sample (200 μ L injected) from a Canavan patient (A) and an age-matched control subject (B). Low but detectable NAA was present in the control sample (sample diluted 25-fold). A large NAA peak in the Canavan patient urinary sample was recorded (sample diluted 400-fold). Neither NAG nor NAAG was detectable in samples from both the Canavan patient and control. Sample processing and chromatographic conditions are described in detail under the Methods section. (1) uracil; (2) β -pseudouridine; (3) hypoxanthine; (4) xanthine; (5) inosine; (6) uric acid; (7) thymidine; (8) orotic acid; (9) methylmalonic acid; (10) *N*-acetylaspartate; (11) 1-methyluric acid.

samples no less than 400 or 10 times, respectively (8 and 2 more times than samples from age-matched control subjects). This high dilution, coupled with the results of co-chromatograms, further rendered the assignment of the NAA peak in Canavan patient samples and strongly supported the present HPLC method as a valid tool for the diagnosis of IEM. Besides showing unusual β -pseudouridine values in these patients, the data summarized in Tables 7 and 8 indicate that the urinary NAA values that we detected in our cohort of Canavan patients were comparable to those previously reported in the literature [12,14,31]. It is however worth emphasizing that, with respect to the methods currently available to measure NAA,

which are mainly based on the use of GC/MS [12,14,31], the present ion-pairing HPLC assay offers the potential to determine not only urinary, but also plasma concentrations of this compound without a differentiated and specific sample preparation procedure. With this in mind, the very simple and time-efficient process of sample deproteinization certainly renders this method even more advantageous when a large number of samples require analysis such as the screening for IEM. According to our results, neither NAG nor NAAG could be found in any of the pathological samples assayed, even when they were injected onto the HPLC column with extra prior dilution (data not shown). This result is in contrast with what was

reported by Burlina et al. [11] but is in accordance with a very recently published paper by Surendrau et al. [32].

In addition to the screening for at least 14 IEM (as listed in Table 1), the present analysis may possibly be applied in several other acute and chronic pathological conditions characterized by alterations of one or more of the compounds considered, such as traumatic head injury, acute myocardial infarction, stroke, Alzheimer's disease, Parkinson's disease, and diabetes. Furthermore, it is also possible to measure GSH, GSSG, ascorbic acid, β -pseudouridine, 3-methyladenine, and 1-methyluric acid, with GSH, GSSG, and ascorbic acid for the monitoring of the oxido-reductive state [33,34], β -pseudouridine, and 3-methyladenine as putative tumor-associated markers [35,36], and 1-methyluric acid as a metabolic index in the caffeine test [37–40]. Taken altogether, these additional applications greatly increase the potential value of the present ion-pairing HPLC method in the clinical biochemistry setting.

Data reported in Table 6 also demonstrate that this technique can be successfully applied to separate and quantify the compounds considered in amniotic fluid specimens. As with plasma, GC/MS fails to detect several compounds related to IEM (NAA, methylmalonic acid, malonic acid, etc.) unless stable isotopic dilution is utilized [6,12,19,31]. This time-consuming, expensive, and complicated method is not regularly used in clinical biochemistry laboratories equipped with GC/MS and is therefore limited to a few specialized centers that chemically diagnose prenatal IEM, with the result of decreasing the number of

Table 8

Purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids in plasma samples of Canavan disease patients determined according to the new ion-pairing HPLC method

	$\mu\text{mol/L}$ plasma
Cytidine	0.26 \pm 0.13
Creatinine	35.23 \pm 5.28
Uracil	2.25 \pm 0.98
Uridine	4.23 \pm 1.92
β -Pseudouridine	16.70 ^a \pm 3.72
Hypoxanthine	5.56 \pm 1.74
Xanthine	6.37 \pm 1.95
Inosine	0.68 \pm 0.47
Ascorbic acid	82.57 \pm 36.71
Uric acid	1797.90 \pm 895.44
Thymidine	0.31 \pm 0.09
Orotic acid	0.94 \pm 0.78
NAA	16.96 ^b \pm 19.57
GSH	30.22 \pm 2.55
GSSG	1.98 \pm 0.41

Values are the mean \pm SD of ten different plasma samples. Sample processing and chromatographic conditions are fully described in the Methods section.

^a Significantly different from value recorded in plasma from control healthy adults; $P < 0.01$.

^b Significantly different from value recorded in plasma from control healthy adults; $P < 0.001$.

Table 9

Purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids in urinary samples of Canavan disease patients determined according to the new ion-pairing HPLC method

	$\mu\text{mol}/\text{mmol}$ creatinine
Cytidine	4.03 \pm 1.77
Uracil	20.24 \pm 7.24
β -Pseudouridine	319.27 ^a \pm 198.36
3-Methyladenine	3.96 \pm 1.44
Hypoxanthine	15.12 \pm 17.44
Xanthine	67.06 \pm 67.87
Inosine	1.00 \pm 1.61
Uric acid	1554.14 \pm 748.85
1-Methyluric acid	7.04 \pm 1.57
Thymidine	4.58 \pm 4.58
Orotic acid	6.11 \pm 5.48
NAA	1872.03 ^b \pm 631.86
Methylmalonic acid	N.D.

Values are the mean \pm SD of ten different urinary samples. Sample processing and chromatographic conditions are fully described in the Methods section.

^a Significantly different from value recorded in urine from age-matched healthy subjects; $P < 0.01$.

^b Significantly different from value recorded in urine from age-matched healthy subjects; $P < 0.001$.

IEM diagnosed in the prenatal stage. Even though we did not find any suspicious case of IEM in the limited number of amniotic fluids that we analyzed, we may affirm that values of the compounds detected in these samples which were characterized by low dispersion, give a strong indication that the sensitivity, reproducibility, and sample preparation of the method are suitable to propose its application in the prenatal chemical diagnosis and screening of IEM.

We can therefore conclude that the ion-pairing HPLC method described here for the separation of purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids with UV detection, characterized by simplicity and time-saving sample processing, as well as reproducibility, sensitivity, and low to moderate instrumentation cost, may represent a valid tool for the screening of a large number of samples for the prenatal and neonatal chemical diagnosis and screening of several important IEM. These characteristics render this ion-pairing HPLC method suitable for use by clinical biochemistry laboratories not only for relevant hospital centers, but also for peripheral hospital structures, thereby increasing the number of laboratories capable of screening for IEM.

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