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Determination by high-performance liquid chromatography with electrochemical detection of free and conjugated N-acetyldopamine excretion in urine of children with neuroblastoma and nephroblastoma

J. Jouve, J. Herault, H. Tournade and J. P. Muh*

Laboratoire de Biochimie Médicale et INSERM U316, CHU de Tours, 2 Boulevard Tonnellé, 37044 Tours Cedex (France)

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ABSTRACT

A simple method for the determination of N-acetyldopamine (NADA) (both free and conjugated) in children's urine by high-performance liquid chromatography with electrochemical detection has been developed. Conjugated NADA was measured as the free compound after enzymatic hydrolysis and purification on alumina. The total analysis time is 25 min. The results show a linearity of the whole assay from 0.005 to 20 μmol/l NADA; the sensitivity is 0.2 pmol per 20 μl injected sample. Mean recoveries of 96.7 and 86.6% were obtained for free and total NADA, respectively. Modifications of the retention times (between 2 and 50 min) induced by changes in the eluent were determined. Conjugated NADA accounted for about 90% of the total excretion of NADA. These results suggest that this compound could play an important part in neuroblastoma; its concentration is thirteen times higher in children with neuroblastoma than in normal subjects.

INTRODUCTION

N-Acetyldopamine (NADA) has long been known to be present in insects and to be involved in the biosynthesis of the cuticle [1]. NADA might also occur in nervous tissues of insects [2], where it presumably has some function related to neural activity [3]. It has been shown that NADA has a significant antitumour activity in experimental leukaemias in mice [4].

The excretion of NADA has been used for the detection of pheochromocytoma [5] and neuroblastoma [6]. The possible significance of NADA in neuroblastoma has received little attention. N-Acetylation may play a significant part in amine metabolism. This is a metabolic route that opens up particularly when the dopamine (DA) concentration is in excess, as in neuroblastoma, or when other routes of amine metabolism are blocked by monoamine oxidase inhibitors [6]. As shown in Fig. 1, N-acetylation could play an essential part in the metabolism of DA and the O-methylation and oxidative deamination processes.

NADA has also been reported to occur in the urine of rats after administration of [^{14}C]DA [7] and was synthesized from L-dihydroxyphenylalanine (L-Dopa) in isolated perfused rat liver [8].

Enzymatic radiochemical [9] and high-performance liquid chromatography (HPLC) with ultraviolet, visible [10] or coulometric detection [3,11,12] assays have been described for the determination of NADA. This compound is present primarily in the conjugated form. The first demonstration of the endogenous occurrence of this compound in urine from normal subjects was reported by Elchisak and Hausner [11]. Since this report, there have been no studies of conjugated NADA. The purpose of this work was to determine the possibility of using the concentrations of free and conjugated NADA in the detection of neuroblastomas and non-secreting neuroblastomas. A simple method is reported here for the

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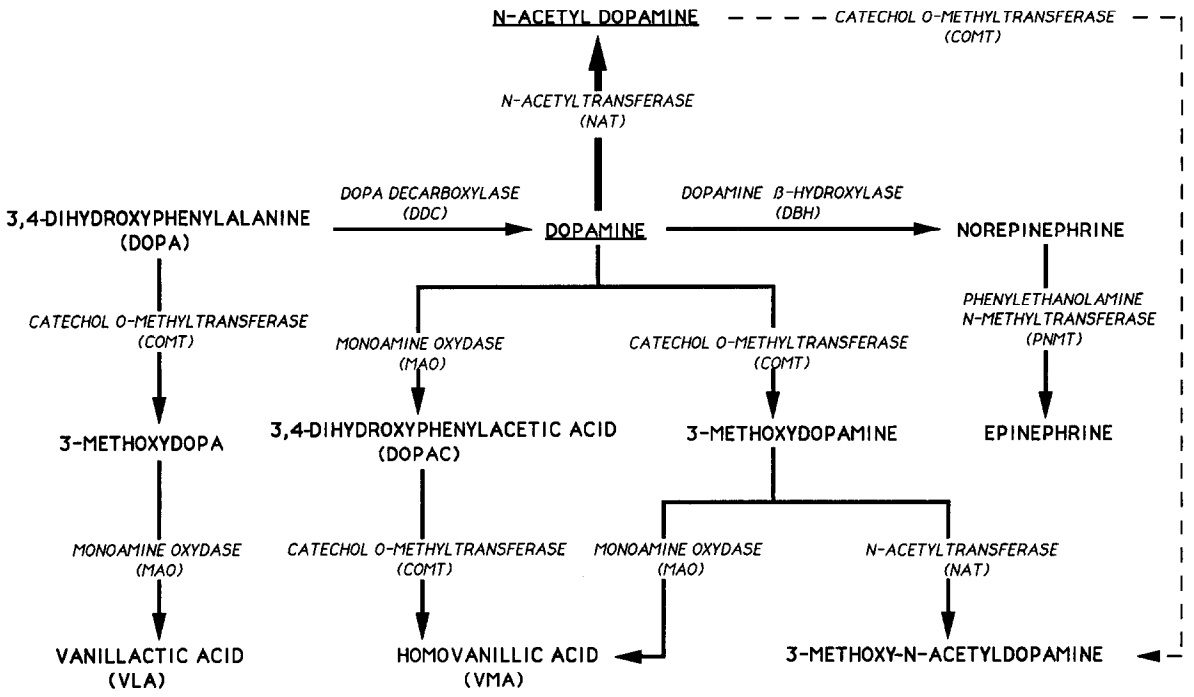

Fig. 1. Pathways of DOPA and DA catabolism.

determination of both free and total NADA in urine. Isocratic HPLC with electrochemical detection (ED), following a one step sample clean-up on alumina [11], yields quantitative recoveries of the compound. HPLC with ED combines high sensitivity, specificity and rapidity sufficient for routine use.

EXPERIMENTAL

Apparatus

A Waters HPLC system was used, equipped with a Model 510 pump, a stainless-steel column (150 mm × 3.9 mm I.D.) packed with Resolve C_{18} (5 μm particle size) and a 460 electrochemical detector (Waters Millipore, Milford, MA, USA). This detector was connected to a CR-5A Shimadzu recorder–integrator (Kyoto, Japan).

Chemicals and enzymes

Ethylenediaminetetraacetic acid disodium salt (EDTA), sodium acetate, sodium disulphite, sodium hydroxide, sodium hydrogenphosphate dihydrate, citric acid, phosphoric acid, mercaptoacetic acid, perchloric acid, acetic acid, tris(hydroxymethyl)aminomethane (Tris) and alumina (90 active neutral), all of analytical-reagent grade, were obtained from Merck (Darmstadt, Germany). Absolute methanol was purchased from Carlo Erba (Milan, Italy), 1-octanesulphonic acid sodium salt (Pic B_8) from Aldrich (Milwaukee, WI, USA) and 3,4-dihydroxybenzylamine hydrobromide (DHBA) and NADA from Sigma (St Louis, MO, USA).

β-Glucuronidase–sulphatase from *Helix pomatia* (EC 3.2.1.31–EC 3.1.6.1) was from IBF Biotecknics (Villeneuve-la-Garenne, France). This mixture of purified enzymes contains 100 000 Fishman units of β-glucuronidase and 1 000 000 Roy units of sulphatase per millilitre. Aqueous solutions were prepared using twice-deionized water from a Millipore system.

Standard solutions

Standard solutions of NADA (512 μmol/l) and DHBA (454 μmol/l) were prepared in 0.01 *M* acetic acid and stored at −4°C. Under these conditions NADA and DHBA remained stable for more than one month.

Chromatographic conditions

The mobile phase was a mixture of 10% (v/v)

methanol in 0.005 *M* citric acid–0.005 *M* Na_2HPO_4 (2:1, v/v) buffer. Sodium octylsulphate and EDTA were added to give final concentrations of $2.5 \cdot 10^{-3}$ and $5 \cdot 10^{-5}$ *M*, respectively. The pH was adjusted to 3.6 with 2 *M* phosphoric acid. Prior to use the mobile phase was filtered through an HVLP 04700 Durapore membrane (Millipore) (pore size 0.45 μm) and with degassed helium.

All separations were carried out at room temperature. During sample injections the flow-rate was usually 0.4 ml/min, and was reduced to 0.1 ml/min when the system was not in use. Once set up, the system was run continuously to ensure stability and sensitivity.

The working electrode potential was 0.7 V *versus* an Ag–AgCl reference electrode.

Collection of urine

A 48-h diet without bananas, pineapple, tomatoes, nuts, chocolate and food containing vanilla was prescribed before urine collection. Three consecutive 24 h urine samples were collected and the volumes recorded. After the addition of sodium disulphite (0.5 g/l) or perchloric acid (pH 3), the urine samples were filtered through Millipore AW 0304700 prefilters (pore size 0.45 μm). They were then stored at −25°C until analysis.

TABLE I

INCUBATION AND EXRACTION PROCEDURE TO PREPARE URINE SAMPLES FOR HPLC ANALYSIS

	NADA	
	Free	Total
Preparation		
Urine sample	2000 μl	2000 μl
Add EDTA (20 g/l)	200 μl	200 μl
Add DHBA (internal standard, 454 μmol/l)	200 μl	200 μl
Add sodium acetate buffer (1 mol/l, pH 4.8)	–	500 μl
Add glucuronidase–sulphatase	–	200 μl
Incubation (37°C, 18 h)		
Stop hydrolysis at 4°C	–	+
	⬇	
Extraction		
Add aluminium oxide	100 mg	
Add mercaptoacetic acid (434 mmol/l)	200 μl	
Add Tris buffer (pH 8.6)	4000 μl	
Adjust to pH 8.6 with sodium hydroxide (0.5 mol/l)		
Shake (15 min), centrifuge (5 min, 3000 *g*, +4°C), discard supernatant		
Washing		
Add 0.2% Tris–EDTA (pH 8.1)	4000 μl	
Shake (3 min), centrifuge (3 min, 3000 *g*, 4°C), discard supernatant		
Repeat washing procedure		
Elution		
Add acetic acid (1 mol/l)	2000 ml	
Shake (10 min), centrifuge (5 min, 3000 *g*, +4°C)		
Dilute supernatant 1:9 (v/v) in mobile phase		
Filter through a Millipore filter (0.45 μm)		
Analysis		
Inject 20 μl on to the HPLC column		

Extraction procedure

The extraction procedures for the urine samples are outlined in Table I. A standard solution (2 ml of 1 *M* acetate buffer, pH 4.8, containing 20.48 nmol of NADA) was extracted with the urine using the same amount of internal standard (90.80 nmol).

RESULTS AND DISCUSSION

Determination of optimum hydrolysis conditions

Two techniques have been developed: perchloric acid hydrolysis and enzymatic hydrolysis.

Perchloric acid hydrolysis. A 2-ml volume of urine was mixed with 10 ml of perchloric acid at different molarities to produce pH values ranging from 1.5 to 3.0. The mixtures were heated at 95°C for 15, 30, 45, 60, 90 and 120 min. The highest concentrations of NADA were obtained when the hydrolysis was conducted at pH 1.8–2.3 In agreement with other workers [11], it was observed that 90 min were necessary to convert conjugated NADA to free catechol. Heating times longer than 90 min resulted in amine loss. The amount of DHBA (90.80 nmol) added to the urine remained constant during this period.

Enzymatic hydrolysis. A 2-ml volume of urine collected on sodium metabisulphite was adjusted to pH 4.8 with perchloric acid incubated with 100, 200, 300 and 400 μl of β-glucuronidase–sulphatase at 37°C for 4, 8, 16, 18, 22 and 24 h. Maximum recoveries of total NADA were obtained when the hydrolysis was conducted with 200 μl of β-glucuronidase–sulphatase for 18 h. The internal standard DHBA (90.80 nmol) was undamaged by the enzymatic procedure.

Some workers have used perchloric acid hydrolysis [11,13,14] and others have chosen enzymatic hydrolysis [15–17] to convert from conjugated to free catechol. A comparison of the results of the two hydrolysis methods shows that the effectiveness of the acid hydrolysis is comparable to the enzymatic hydrolysis. With acid hydrolysis, a visible interference with the NADA peak can be seen. Consequently, enzymatic hydrolysis was preferred for this assay. Chromatograms of standard solutions before and after hydrolysis indicated that there was no breakdown of NADA during the enzymatic hydrolysis procedure (Fig. 2A and B).

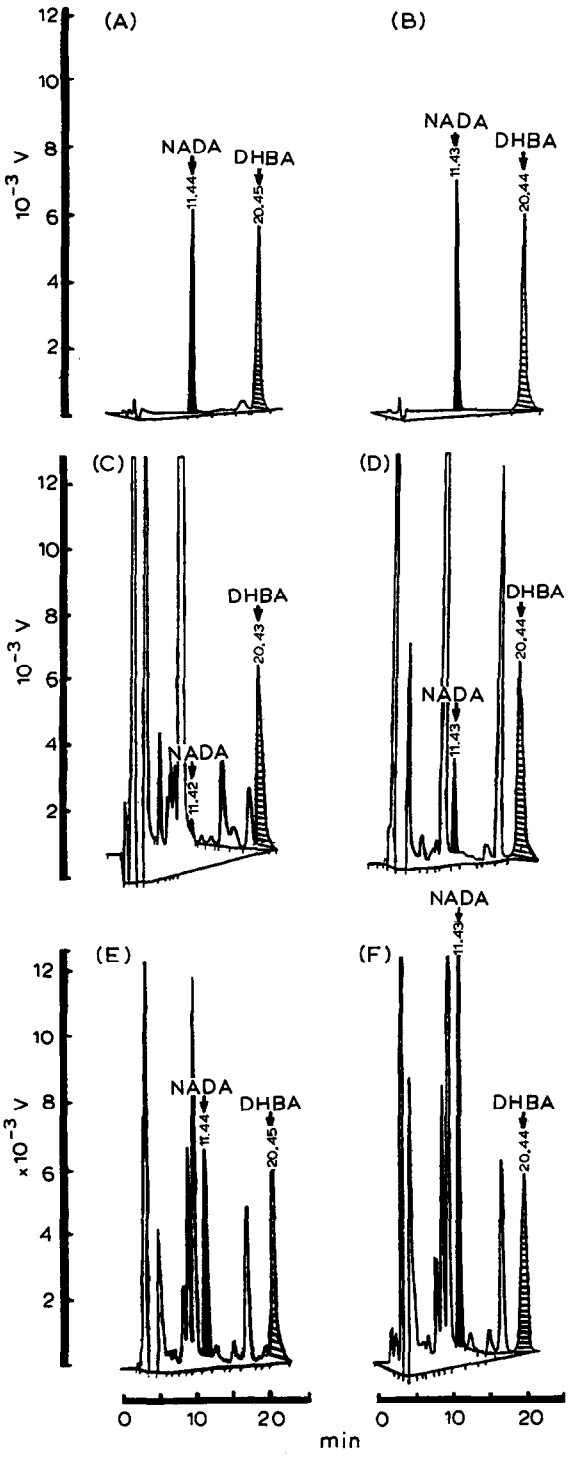

Fig. 2. Chromatograms obtained after enzymatic hydrolysis. (A) Unhydrolysed and (B) hydrolysed standard mixture of 20.48 nmol NADA and 90.8 nmol DHBA; (C) unhydrolysed and (D) hydrolysed urine sample from a nine-year-old, normal child; (E) unhydrolysed and (F) hydrolysed urine sample from a nine year old child with neuroblastoma.

TABLE II

ANALYTICAL RECOVERY OF FREE AND TOTAL NADA

Sample	Concentration (nmol/l)			Recovery[a] (%)
	Present	Added	Found	
NADA in unhydrolysed urine (n = 5)				
Urine 1	93	512	585	96.7
Urine 2	140	1024	1115	95.8
Urine 3	229	1536	1722	97.6
NADA in hydrolysed urine (n = 5)				
Urine 1	940	512	1254	86.3
Urine 2	3100	1024	3509	85.1
Urine 3	4363	1536	5220	88.5

[a] Ratio of (amount found after spiking)/(amount before spiking + amount added in spike).

Recovery

To determine the recoveries of the entire procedure for the determination of free and total NADA, five aliquots of three different urine samples alone or spiked with different amounts of NADA were analysed. The results are summarized in Table II. The mean recovery of NADA added to unhydrolysed urine was 96.7% and the recovery of NADA added to hydrolysed urine was 86.6%.

Precision

The day-to-day precision was determined from an analysis of a urine pool from normal children over a period of fifteen days. Within-batch precision was evaluated by analysing the pooled urine twenty consecutive times. The data in Table III show variations of less than 15% for the intra-assay precision and less than 16% for the inter-assay precision.

Limit of detection and linearity

The detection limit calculated from the peak height equivalent to 2% of full scale was 0.2 pmol for NADA. The linearity of the response was investigated for urine samples after the addition of increasing amounts of NADA (0.01–40 nmol) to 2 ml of urine. At each point, triplicate determinations were carried through the hydrolysis and assay procedure. A linear relationship between the NADA concentration and the peak height was observed over the concentration ranges studied. The equation for the calibration curve was $y = 0.049x - 0.006$ and the coefficient of correlation (r) was 0.99983.

Quantification

The concentration of NADA in each sample was calculated by determining the peak-area ratio relative to DHBA, and comparing these with those obtained with synthetic standards.

Specificity

The NADA peak was identified from the retention times of the standard and the sample peak after modification of the various components of the mobile phase and after varying the oxidation potential between 0.5 and 1 V. None of the possible interfering compounds such as 3,4-dihydroxyphenylalanine and N-acetylnorepinephrine [3], which might be carried through the alumina extraction procedure, interfered with the NADA peak.

Optimization of the chromatographic conditions

The mobile phase must be carefully fine-tuned to prevent interfering peaks. It is necessary to test the action of a number of factors such as pH, methanol concentration, 1-octanesulphonic acid

TABLE III

PRECISION OF THE METHOD

Compound	Intra-assay (n = 20)		Inter-assay (n = 15)	
	Concentration (mean ± S.D.) (nmol/l)	C.V. (%)	Concentration (mean ± S.D.) (nmol/l)	C.V. (%)
Free NADA	154 ± 14.3	9.3	191 ± 23	12.1
Total NADA	2979 ± 43.2	14.5	3115 ± 49.5	15.9

(Pic B_8) concentration and phosphate and citrate concentrations [18,19] to give the desired modification of retention times. We studied the parameters that affect the retention times in this type of chromatography.

Increasing the pH value had a minor effect on the retention times. A low pH should be used to obtain a good separation of all substances. The pH of the mobile phase was adjusted to 3.6 (see earlier). At the pH values studied here, the retention times of NADA and DHBA were decreased on the addition of methanol. Increasing Pic B_8 and decreasing the phosphate concentrations greatly increased the retention times of DHBA, but there was no significant change for NADA. The citrate concentrations did not affect the retention times of these compounds.

Adequate chromatographic resolution, together with a reasonable analysis time, was obtained with this mobile phase. The chromatograms (Fig. 2) show that NADA was well separated from numerous catechol compounds or derivatives that might also occur in human urine.

An electrode potential of 0.7 V *versus* Ag–AgCl provided sufficient sensitivity for the determination of picomole amounts of NADA with minimum interference from solvent effects and electrical noise. Higher potentials (0.8–1 V) favour the oxidation of several compounds, including monophenols, and increase the number of possible interferences.

Reference values

Urine samples were obtained from 40 healthy children, aged 0–15 years, who followed the dietary conditions stated earlier. Medication was discontinued. Typical chromatograms of an unhydrolysed and hydrolysed urine sample from a normal child are illustrated in Fig. 2.

The reference values were (mean ± S.D.): free NADA, 0.0293 ± 0.0125 μmol/mmol of creatinine; total NADA 0.389 ± 0.285 μmol/mmol of creatinine. Conjugated NADA (difference between total and free) accounted for 92.4% of the total NADA excretion. These results are in agreement with those obtained elsewhere [11] and clearly indicate that conjugation is a significant metabolic pathway for NADA in humans.

Pathology

The urinary concentrations of free and total NADA were determined in children with neuroblastoma and nephroblastoma prior to treatment to evaluate the usefulness of these parameters for the diagnosis of neuroblastoma. Increased concentrations were seen in children with neuroblastoma (thirteen times more than normal values; Table IV) and in children with nephroblastoma (four times higher than normal). In non-secreting neuroblastoma, the non-differentiated tumour cells do not secrete the usual catecholamines, vanillylmandelic acid, homovanillic acid, norepinephrine, epinephrine and DA, but only L-dopa

TABLE IV

FREE AND CONJUGATED NADA EXCRETION IN CHILDREN WITH NEUROBLASTOMA AND NEPHROBLASTOMA

Values are given as μmol/mmol creatinine. Values (mean ± S.D.) for normal patients (0–15 years, n = 40): free NADA, 0.0293 ± 0.0125; total NADA, 0.389 ± 0.285; conjugated NADA, 92.4%.

Disease	Sex	Age	Free NADA	Total NADA	Conjugated NADA (%)
Neuroblastoma stage IV	M	8 days	0.477	10.140	99.5
Neuroblastoma stage III	M	15 days	0.254	5.691	95.5
Neuroblastoma stage III	M	9 years	0.693	5.660	87.7
Neuroblastoma stage IV, relapse after surgery and chemotherapy	F	13 years	0.570	3.163	81.9
Neuroblastoma stage III, relapse after surgery and chemotherapy	M	9 years	0.222	2.969	92.5
Thoraco abdominal neuroblastoma	M	18 months	0.118	1.880	93.7
Nephroblastoma	M	1 year	0.123	1.919	85.5
Nephroblastoma	F	5 years	0.192	1.090	82.3
Nephroblastoma	M	1 year	0.098	0.846	88.4

and its metabolite vanillactic acid [22]. It is possible that L-dopa was also metabolized to NADA [8]. In neuroblastoma, considered to be metabolically inactive, when the tumour cells are immature N-acetylation might be a by-pass of metabolism, more prominent than other metabolic pathways (O-methylation, deamination). Different N-acetylated amines have also been detected in urine from patients with neuroblastoma, such as N-acetylnormetanephrine and N-acetylmetanephrine [23]. In addition, aliphatic amino acids such as cystathionine can be acetylated [15]. It is possible that the presence of DA in large amounts favours this metabolic route. However, the determination of free and conjugated NADA could be used as a marker together with vanillylmandelic acid, homovanillic acid, norepinephrine, epinephrine, DA and methoxyamines for the management and diagnosis of neuroblastoma.

These markers, with vanillactic acid assay, could be useful when surgical indications are uncertain and to supply complementary information to determine the difference between neuroblastoma nad nephroblastoma.

REFERENCES

1 S. O. Andersen, *Ann. Rev. Entomol.*, 24 (1979) 29.
2 S. Hayashi, L. L. Murdock and E. Florey, *Comp. Biochem. Physiol. C*, 58 (1977) 183.
3 D. Omar and L. L. Murdock, *J. Chromatogr.*, 224 (1981) 310.
4 M. M. Wick and A. Mui, *J. Natl. Cancer Inst.*, 66 (1981) 351.
5 E. Sekeris and P. Herrlich, *Hoppe-Seylers Z. Physiol. Chem.*, 331 (1963) 289.
6 A. Hanson and W. Von Studnitz, *Clin. Chim. Acta*, 11 (1965) 384.
7 M. Goldstein and J. M. Musacchio, *Biochim. Biophys. Acta*, 58 (1962) 607.
8 G. M. Tyce, *Biochem. Pharmacol.*, 20 (1971) 3447.
9 M. W. McCaman, R. E. McCaman and J. Stetzler, *Anal. Biochem.*, 96 (1979) 175.
10 S. J. Saul and M. Sugumaran, *FEBS Lett.*, 251 (1989) 69.
11 M. A. Elchisak and E. Hausner, *Life Sci.*, 35 (1984) 2561.
12 R. G. H. Downer and R. J. Martin, *Life Sci.*, 41 (1987) 833.
13 M. A. Elchisak and J. H. Carlson, *J. Chromatogr.*, 233 (1982) 79.
14 N. D. Vlachakis, E. Kegosov, S. Yoneda, N. Alexander and R. F. Maronde, *Clin. Chim. Acta*, 137 (1984) 199.
15 P. Mathieu, J. Greffe, D. Dernaz, R. Guilluy and L. Gjessing, *Biochem. Pharm.*, 25 (1976) 497.
16 J. Claustre, P. Serusclat and L. Peyrin, *J. Neural Transm.*, 56 (1983) 265.
17 B. Sjöquist, H. Axson Jonnson and S. Borg, *Drug Alcohol Depend.*, 16 (1985) 241.
18 D. Afonso, M. A. Castellanos and M. Rodriguez, *J. Chromatogr.*, 528 (1990) 101.
19 R. Pagliari, J. M. Cottet-Emard and L. Peyrin, *J. Chromatogr.*, 563 (1991) 23.
20 T. Philip, B. Kremens and J. P. Chappuis, *Pediatrics*, 42 (1987) 143.
21 T. Philip, B. Kremens and J. P. Chappuis, *Pediatrics*, 42 (1987) 231.
22 J. Jouve, D. Bakri, J. Herault and J. P. Müh, *J. Chromatogr.*, 567 (1991) 331.
23 O. Borud and L. R. Gjessing, *Clin. Chim. Acta*, 27 (1970)552.