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Quantitation of tryptophan and other plasma amino acids by automated pre-column *o*-phthaldialdehyde derivatization high-performance liquid chromatography: improved sample preparation

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ABSTRACT

Pre-column derivatization with *o*-phthaldialdehyde is a rapid and sensitive method for the quantitation of amino acids in biological fluids. This method uses acetonitrile as a deproteinizing reagent which gives improved recovery of tryptophan compared with 5-sulfosalicylic acid and permits the measurement of aspartic acid which coelutes with 5-sulfosalicylic acid. The method is automated to increase reproducibility and convenience. Mean coefficients of variation for peak areas relative to internal standard were 3.2 and 5.2% for amino acid standards and plasma samples, respectively. The presence of nitrilotriacetic acid stabilized the *o*-phthaldialdehyde reagent which is important in an automated system. The method is suitable for the analysis of large numbers of plasma samples where total tryptophan and aspartic acid are of interest.

INTRODUCTION

A range of techniques is currently available for measuring amino acids in biological fluids including the classical method of ion-exchange chromatography followed by ninhydrin derivatization [1] and derivatization by *o*-phthaldialdehyde (OPA), dansyl chloride, dabsyl chloride or phenylisothiocyanate [2,3]. Each of these methods has its limitations. The classical method is restricted by its long analysis time, limited sensitivity and high capital and operational cost. The dansyl and dabsyl chloride and phenylisothiocyanate derivatizations suffer from long and complex reaction procedures. We required a method which would allow the rapid quantitation of the amino acid profiles of a large number of plasma samples with a high degree of reproducibility and decided that pre-column derivatization using OPA–2-mercaptoethanol (2-ME) would be the most appropriate method to use. Post-column derivatization with OPA is slower than pre-column derivatization and more complicated due to the extra pumps required for the derivatization. OPA reacts with primary amines in the presence of 2-ME to produce strong-

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ly fluorescent products [4]. The derivatized amino acids (isoindoles) can then be separated on a reversed-phase column by high-performance liquid chromatography (HPLC) and quantified using fluorescence detection. This method is capable of separating and quantifying most amino acids present in plasma (with the exceptions of the imino acids proline and hydroxyproline), is relativley quick (40–60 min) and is readily automated [2–3,5–14].

Two problems of OPA pre-column derivatization of biological fluids are reported in the literature. Firstly, that the aspartic acid derivative coelutes with the commonly used deproteinizing agent 5-sulfosalicylic acid (SSA) and, secondly, that recovery of tryptophan is incomplete using SSA [15–17], necessitating the use of separate assays for one or both of these amino acids. To resolve these problems an alternative method of deproteinizing plasma was needed. Acetonitrile has been used by other workers [5,6,15], but has not been investigated fully. It does not interfere with the measurement of aspartic acid [8,9] and has been reported to give full recovery of tryptophan [15].

A manual method [6,9,14,18] of pre-column derivatization was set up, and then modified to enable the derivatization to be carried out automatically. In optimizing this method we investigated: (1) the different methods of sample preparation (protein precipitation by SSA, trichloroacetic acid and acetonitrile); (2) the applicability of several columns; (3) the optimal time for reaction; (4) the addition of surfactant (Brij 35) and metal chelating agent (nitrilotriacetic acid) to the OPA derivatization reagent; (5) the recovery of amino acids from plasma; (6) the reproducibility of the method.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of two high-pressure delivery pumps, an M6000 (pump A) and an M45 (pump B), a WISP 710B autosampler, a Model 721 system controller, a Model 730 data module, a Model 420 AC fluorescence detector (with an 8-μl flow cell, 338-nm excitation and 425-nm emission filters) and a column heater (sets at 35°C) all supplied by Waters Assoc. (Milford, Massachusetts, U.S.A.).

The separation of amino acid derivatives was performed using the following reversed-phase columns: LiChrospher 100 C_{18} (5 μm, 125 mm × 4.6 mm I.D.); LiChrospher 100 C_{18} (5 μm, 250 mm × 4.6 mm I.D.) both from Merck (Darmstadt, F.R.G.) and Dynamax Microsorb C_{18} (5 μm, 150 mm × 4.6 mm I.D.) from Rainin Instruments (Woburn, MA, U.S.A.). The Rainin column has proved to be the most suitable for this application giving very good separation and considerably longer column lifetime in the order of 600 runs compared with 140 and 250 runs for previous columns.

A LiChrospher 100 RP-18 guard column, 5 μm (Merck), was used to protect the analytical column. A microvolume mixer (a guard column filled with 600-μm

glass beads) supplied by Waters Assoc. was inserted between the WISP and the column in the automated system.

Reagents

Deionized water (LiquiPure Modulab) was used for the preparation of buffers. The chemicals were of analytical grade or better and the solvents of HPLC grade. Sodium acetate boric acid, methanol, sodium hydroxide, acetic acid, potassium monohydrogenphosphate, phosphoric acid, nitric acid and acetonitrile 190 were purchased from Ajax Chemicals (Auburn, Australia). Iodoacetic acid and OPA were purchased from Merck; homoserine, ornithine, carnosine and taurine from Sigma (St. Louis, MO, U.S.A.); trichloroacetic acid (TCA), 2-ME, SSA and nitrilotriacetic acid (NTA) from BDH (Poole, U.K.). Amino acid standard H and Brij 35 (30% solution) were purchased from Pierce (Rockford, IL, U.S.A.) and tryptophan, citruline, alanine, arginine, asparagine, aspartic acid, cystine, glutamic acid, glutamine, glycerine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, valine, 1-methylhistidine, 3-methylhistidine, α-aminobutyric acid, γ-aminobutyric acid from Fluka (Buchs, Switzerland).

Sodium acetate buffer (0.1 *M*) was prepared by dissolving sodium acetate in water and titrating to pH 6.8 with acetic acid. Sodium borate buffer (0.4 *M*) was prepared by dissolving boric acid in water and titrating to pH 9.5 with sodium hydroxide solution. Potassium phosphate (0.1 *M*) was prepared by dissolving potassium monohydrogenphosphate in water and titrating to pH 4 with phosphoric acid. Iodoacetic acid reagent (3.7%) was prepared by dissolving iodoacetic acid in the borate buffer.

Mobile phase A, methanol–sodium acetate buffer (95 ml methanol made up to 100 ml with sodium acetate buffer), and mobile phase B, methanol–sodium acetate (95 ml acetate buffer made up to 100 ml with methanol), were filtered and degassed by passing through a 0.22-μm Durapore Millipore filter (Milford, MA, U.S.A.) prior to use and thereafter at the start of each day.

Plasma collection

Blood was collected from the antecubital vein of healthy subjects in the morning after an overnight fast. Samples were collected in lithium heparin tubes. Plasma was separated by centrifugation (2000 *g* for 20 min at 4°C). Plasma samples were then used immediately or stored at −70°C.

Sample preparation

Amino acid standard. Homoserine (internal standard), tryptophan, glutamine, asparagine, taurine and ornithine were added to Pierce amino acid standard H, diluted to 2.5 μmol/ml with water and stored at −20°C until use; this mixture was used as amino acid standard. The standard was diluted 1:100 with protein precipitant (acetonitrile–2-ME, 10 ml/20 μl); 5 μl of amino acid standard were used in the assay.

Plasma samples. Homoserine (5 μl; 2.5 mmol/ml) was added as an internal standard to 100 μl of plasma and made up to 500 μl with acetonitrile–2-ME protein precipitant in a 1.5-ml Eppendorf centrifuge tube. The tube was vortexed and centrifuged for 4 min in a Beckman microfuge. The supernatant was then removed and either used immediately or stored in a 1.5-ml Eppendorf centrifuge tube at −70°C until analysed. Plasma samples were also deproteinized with SSA and TCA in which case 5 mg of SSA in 400 μl of borate buffer or 100 μl of TCA (10%) in 300 μl of borate buffer replaced the acetonitrile–2-ME.

Peak identification

Amino acid derivatives (from the amino acid standard and the plasma samples) were identified by comparing retention times with those obtained from individual amino acids and also spiking samples with amino acids.

Quantification of amino acids

The amino acid standard was chromatographed twice and thereafter every fifth analysis in any automated series. All amino acid standards and plasma samples contained the internal standard (I.S.) homoserine. The concentration of amino acids (AA) in plasma samples could then be determined.

$$\text{AA } (\mu M) = \frac{\text{AA area} \times 125/\text{AA area in standard} \times \text{I.S. area in AA standard}}{\text{I.S. area}}$$

The concentrations of amino acids were calculated using a computer spreadsheet (OPEN ACCESS 3 SPI).

Derivatization

The derivatization reagent was prepared by modifying the method developed by Jones *et al.* [12]. OPA (50 mg) was dissolved in methanol (1 ml) and added to borate buffer (11 ml), 2-ME (50 μl) and nitrilotriacetic acid (10 mg). For a trial period Brij 35 (200 μl) was also added. The reagent was then filtered (0.2 μm FlowPore filter) and stored in the dark at 4°C. 2-ME (20 μl) was added weekly to maintain the level of 2-ME in the derivatization reagent.

When the manual derivatization procedure was used, amino acid standard (40 μl) or deproteinized plasma (40 μl) was mixed with OPA derivatizing reagent (40 μl) and with indoleacetic acid solution (20 μl) which carboxymethylates cysteine allowing its detection [12] (it was too complicated to include this step in the automated system). After a reaction time of 1 min the volume was made up to 200 μl with potassium phosphate buffer and 20 μl of the solution were injected onto the column immediately after mixing.

The automated method was adapted from Waters AUTO TAG OPA precolumn derivatization technique [11]. Derivatization reagent (approximately 1 ml) was put in a sample vial with a self-sealing septum and placed in the first position

in the autosampler. Amino acid standards and deproteinized plasma samples were placed in 150-μl glass limited-volume inserts (Waters Assoc.), inside sample vials in subsequent positions in the autosampler. The derivatizing reagent (5 μl) was injected onto the mixing column with the HPLC system flow-rate at 0.05 ml/min. After 1 min, 5 μl of the sample to be analysed were injected onto the mixing column. After 2.5 min mixing time, the flow-rate was increased to 1.5 ml/min and derivatized amino acids were carried to the column. After the analysis the autosampler returned automatically to the first position in the rack, injected derivatizing reagent, moved on to the second sample and so on. The system could be run unattended for longer than 24 h and continuously for several days.

Chromatographic conditions

The gradient used in the analysis (Table I) has been developed to optimize the separation of glycine/threonine, tryptophan/methionine and glutamine/histidine which have been reported to coelute under similar conditions [8–10,12]. Acetonitrile was not added as an organic modifier to the solvent system [8] as the gradient developed enabled baseline resolution of all amino acid derivatives.

RESULTS AND DISCUSSION

Recovery of amino acids from plasma

Plasma samples were spiked with known amounts of amino acids (250 pmol). These were then analysed and compared with the appropriate plasma sample and

TABLE I

CHROMATOGRAPHIC GRADIENT PROGRAM USED IN THE ANALYSIS

Time (min)	Flow-rate (ml/min)	Percentage solvent A	Curve	Comment
0	0	15	6	Inject OPA
1		15		Inject sample
3	0.05	15	1	
3.5	1.5	15	6	Mixing complete
19	1.5	30	6	
28	1.5	55	6	
30	1.5	60	6	
38	1.5	100	6	
41	1.5	100	6	Wash
45	1	0	6	
48	1	0	6	Wash
49	1	15	6	Re-equilibrate
51	1	15	6	
53	0	15	6	Initial conditions

TABLE II

RECOVERY AND LINEARITY OF RESPONSE

Amino acid	Recovery[a] (%)	Linearity of response[b]
Aspartic acid	89	0.997
Glutamic acid	95	0.999
Asparagine	97	0.999
Serine	97	0.999
Glutamine	113	0.993
Histidine	104	0.960
Glycine	104	0.999
Threonine	100	0.999
Arginine	95	0.986
Taurine	105	0.999
Alanine	103	0.999
Tyrosine	100	0.999
Tryptophan	102	0.998
Methionine	103	0.999
Valine	97	0.999
Phenylalanine	96	0.999
Isoleucine	99	0.999
Leucine	101	0.999
Lysine	85	0.935

[a] Recovery of 250 pmol of amino acid standard from plasma.
[b] Linear regression coefficients (amino acid standard 31–500 pmol).

amino acid standard. The recovery of each amino acid (the plasma sample plus amino acid standard divided by the spiked plasma sample) was expressed as percentage recovery (Table II). Of these values only aspartic acid, present in very low levels in plasma, and lysine, which has a relatively low and variable color yield, gave values below 100%.

Linearity of response

Amino acids with concentrations ranging from 31 to 500 μM were chromatographed. The response of these amino acids was compared with their known concentration. The response of the system was linear for a range of amino acid concentrations (31–500 μM) with linear regression coefficients ranging from 0.999 to 0.935 and averaging 0.992 (Table II).

Comparison of different methods of deproteinization

In blood, tryptophan is found mostly bound to albumin with only a small percentage free [16,19]. As a result it is important to know if free, total or some

TABLE III

METHODS OF DEPROTEINIZATION

Amino acid	Concentration (μM)		
	Acetonitrile[a]	TCA	SSA
Aspartic acid	4	2	3
Glutamic acid	20	26	28
Asparagine	93	74	78
Serine	103	116	111
Glutamine	580	574	597
Histidine	96	72	84
Homoserine	125	125	125
Glycine	235	278	290
Threonine	176	144	152
Arginine	58	116	111
Taurine	26	25	24
Alanine	378	332	357
Tyrosine	78	57	61
Tryptophan	107	82	87
Methionine	40	31	34
Valine	272	181	194
Phenylalanine	87	60	63
Isoleucine	85	60	67
Leucine	185	141	151
Ornithine	55	116	111
Lysine	90	209	213

[a] Mean of two determinations.

fraction of total tryptophan is being measured. It has been reported [15] that acetonitrile deproteinization results in full recovery of total tryptophan while SSA deproteinization results in incomplete [15,17] or variable [16] recovery. For this reason the acetonitrile method of deproteinization was selected. Qureshi and Qureshi [20] compared different methods of deproteinization including SSA and acetonitrile but unfortunately did not separate tryptophan.

The efficacy of acetonitrile, TCA and SSA as deproteinizing agents were compared (Table III). A higher recovery of tryptophan was found with acetonitrile relative to TCA and SSA in agreement with others [15–17]. The concentrations of arginine and lysine measured using acetonitrile were lower than those measured with SSA and TCA. Problems with the fluoresence of lysine have been reported by others [2,7–9,13]. The high values for ornithine with TCA and SSA are probably due to the very poor colour yield associated with this amino acid (approximately one tenth of most others). For this reason values for ornithine have not been included in our discussion. Qureshi and Qureshi [20] also reported greater

TABLE IV

REPRODUCIBILITY

Amino acid	Retention time (n = 14)			Relative peak area					
	Mean (min)	S.D. (min)	C.V. (%)	Standard[a] (n = 14)			Plasma[b] (n = 12)		
				Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)
Aspartic acid	6.37	0.02	0.3	0.99	0.03	3.1	0.03	0.00	7.6
Glutamic acid	7.72	0.03	0.4	1.06	0.02	1.8	0.14	0.01	5.2
Asparagine	10.81	0.07	0.6	0.56	0.03	5.9	0.34	0.02	6.0
Serine	12.25	0.08	0.6	1.23	0.05	3.8	1.04	0.06	5.5
Glutamine	14.20	0.01	0.7	1.02	0.02	2.3	5.01	0.21	4.2
Histidine	15.11	0.11	0.8	0.27	0.02	7.7	0.34	0.01	3.5
Homoserine	16.31	0.11	0.7						
Glycine	17.75	0.25	1.4	1.00	0.03	2.9	2.01	0.09	4.6
Threonine	18.56	0.12	0.7	0.76	0.02	3.0	1.21	0.04	3.1
Arginine	20.83	0.16	0.8	0.71	0.01	2.0	0.34	0.01	2.0
Taurine	22.60	0.16	0.7	2.27	0.04	1.6	0.50	0.04	7.2
Alanine	24.58	0.13	0.5	0.88	0.03	4.3	2.96	0.17	5.7
Tyrosine	25.88	0.11	0.4	0.82	0.01	1.6	0.70	0.06	8.2
Tryptophan	31.25	0.07	0.2	0.56	0.02	3.9	0.59	0.03	5.9
Methionine	31.41	0.07	0.2	0.90	0.01	1.2	0.31	0.02	7.7
Valine	31.90	0.06	0.2	0.98	0.02	1.8	2.33	0.12	3.4
Phenylalanine	32.75	0.10	0.3	0.69	0.02	3.2	0.57	0.04	7.1
Isoleucine	34.31	0.05	0.2	0.97	0.01	1.6	0.71	0.02	3.4
Leucine	34.88	0.05	0.1	0.71	0.03	3.7	1.34	0.09	6.6
Lysine	36.92	0.03	0.1	0.47	0.04	8.0	0.32	0.01	4.1

[a] Peak area in amino acid standard relative to the internal standard homoserine.

[b] Peak area in plasma sample relative to the internal standard.

degradation of amino acids in organic solvents compared to SSA and increased aspartic acid and glutamic acid levels due to hydrolysis of asparagine and glutamine, respectively, in acetonitrile compared with SSA. We did not observe such effects.

Godel *et al.* [7] suggested higher levels of amino acids after acetonitrile deproteinization reported by some workers [8,9] were due to proteolysis of albumin. To investigate this possibility we deproteinized a solution of bovine serum albumin (7%) but failed to detect measurable levels of amino acids.

Reproducibility

Replicate injections of amino acid standard (n = 14) and of plasma (n = 12) were analysed (Table IV). The retention times showed very little variation with

TABLE V

COMPARISON WITH LITERATURE VALUES

Amino acid	Concentration (μM)			
	Literature[a]		This paper[b]	
	Mean	Range	Mean	S.D.
Aspartic acid	37	3–146	7	2
Glutamic acid	71	21–230	10	6
Asparagine	57	36–87	78	34
Serine	118	83–157	89	32
Glutamine	611	448–1029	619	98
Histidine	90	62–150	70	12
Glycine	249	197–320	199	48
Threonine	134	99–190	163	44
Arginine	86	55–118	34	10
Taurine	91	29–203	62	26
Alanine	347	270–447	361	69
Tyrosine	67	52–83	75	16
Tryptophan	52	31–64	76	19
Methionine	26	16–37	29	7
Valine	231	188–287	271	32
Phenylalanine	63	45–98	71	15
Isoleucine	68	39–87	74	17
Leucine	139	115–193	163	32
Lysine	180	135–243	63	16

[a] Taken from refs. 5,7,9,20 and 23–33.
[b] $n = 11$.

coefficients of variation values ranging from 0.01 to 1.4% with a mean of 0.48%. The relative peak areas (peak area relative to the internal standard, as used in calculating the concentration of amino acids in samples) had coefficients of variation ranging from 1.3 to 8.0% with a mean of 3.2%. As would be expected, in plasma peak areas showed greater variability: 2.0–7.6% with a mean of 5.2%.

Derivatization

The addition of nitrilotriacetic acid to the OPA reagent has been reported [21] to reduce the oxidation of 2-ME and thus improve its stability by acting as a metal chelating agent. After the addition of nitrilotriacetic acid we observed a dramatic increase in the stability of the OPA reagent (with the reagent stability in the sample vial increased at least four-fold) for this reason it was included in our derivatization reagent. Brij 35 has been reported to increase the fluorescence of the lysine-OPA derivative [22]; however, we did not detect any improvement with its addition to our derivatization reagent.

Comparision with other techniques

Fasting plasma samples were taken from eleven healthy subjects and mean amino acid levels determined to compare the method we have developed with the values measured by a range of other workers [5,7,9,20,23–33] (Table V).

The levels we obtained for glutamic acid, arginine and lysine were low while those for tryptophan and ornithine were high compared with the mean literature values. The reason for lower values for glutamic acid and arginine is unclear; however, there is considerable variation in amino acid levels amongst the population, dependent upon a whole range of variables including age, nutritional status and time of day. There is also considerable variation in the literature with glutamic acid in particular with published mean values ranging from 21 to 230 μM.

CONCLUSIONS

In conclusion we have reported a reliable method for quantifying amino acids in plasma. In particular, this method gives good recoveries of tryptophan, an amino acid of particular interest in our laboratory and one which does not result in good recoveries with SSA deproteinization. We were able to separate and quantify more than 23 amino acids in plasma samples in less than 40 min with a total run time of under 1 h. The method is automated to increase reproducibility and convenience. The reproducibility is satisfactory with mean coefficients of variation of 3.2 and 5.2% in standard and plasma, respectively, for peak areas relative to the internal standard. The presence of nitrilotriacetic acid stabilized the OPA reagent which is critical in an automated system where the reagent needs to be stable for 24–48 h.

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REFERENCES

1 S. Moore, D. H. Spackman and W. H. Stein, *Anal Chem.*, 30 (1958) 1185.
2 G. McClung, W. T. Frankenberger, *J. Liq. Chromatogr.*, 11 (1988) 613.
3 P. Furst, L. Pollack, T. A. Graser, H. Godel and P. Stehle, *J. Chromatogr.*, 499 (1990) 557.
4 M. Roth, *Anal. Chem.*, 43 (1971) 880.
5 H. M. H. van Eijk, M. A. H. van der Heijden, C. L. H. van Berlo and P. B. Soeters, *Clin. Chem.*, 34 (1988) 2510.
6 P. Lindroth and K. Mopper, *Anal. Chem.*, 51 (1979) 1667.
7 H. Godel, T. Graser, P. Foldi, P. Pfaeder, P. Furst, *J. Chromatogr.*, 297 (1984) 49.
8 B. N. Jones and J. P. Gillian, *J. Chromatogr.*, 266 (1983) 471.
9 D. C. Turnell and J. D. H. Cooper, *Clin. Chem.*, 28 (1982) 527.
10 R. J. Smith and K. A. Pianco, *J. Liq. Chromatogr.*, 8 (1985) 1783.

11 *Waters AUTO TAG OPA Precolumn Derivatization Technique,* Waters Technical Bulletin, Waters Assoc., Milford, MA, November 1982.
12 B. N. Jones, S. Paabo and S. Stein, *J. Liq. Chromatogr.,* 4 (1981) 565.
13 G. A. Qureshi, L. Fohlin and J. Bergstrom. *J. Chromatogr.,* 297 (1984) 91.
14 R. Schuster, *J. Chromatogr.,* 431 (1988) 271.
15 R. E. Kelley, R. P. Lavan, C. Yen, L. DiMeglio and M. C. Storm, *J. Parenter. External Nutr.,* 10 (1986) 19S.
16 A. Mondino, G. Bongiovanni and S. Fumero, *J. Chromatogr.,* 104 (1975) 297.
17 R. W. Hubbard, J. G. Chambers, A. Sanchez, R. Slocum and P. Lee, *J. Chromatogr.,* 431 (1988) 163.
18 E. Luke and B. Parker, unpublished results.
19 R. H. McMenamy, C. C. Lund and Y. L. Oncley, *J. Clin. Invest.,* 36 (1957) 1672.
20 G. A. Qureshi and A. R. Qureshi, *J. Chromatogr.,* 491 (1989) 281.
21 M. E. May and L. L. Brown, *Anal. Biochem.,* 181 (1989) 135.
22 J. R. Benson and P. E. Hare, *Proc. Natl. Acad. Sci. U.S.A.* 72 (1975) 619.
23 C. R. Scriver, D. M. Gregory, D. Sovetts and G. Tissenbaum, *Metabolism,* 34 (1985) 868.
24 M. D. Armstrong and U. Stave, *Metabolism,* 22 (1973) 549.
25 T. L. Perry and S. Hansen, *Clin. Chim. Acta,* 25 (1969) 53.
26 M. Beylot, C. Chambier, A. Moneger and R. Cohen, *Diabetes Metab.,* 15 (1989) 38.
27 A. Montanari, I. Simoni, D. Vallisa, A. Trifiro, R. Colla, R. Abbiati, L. Borghi and A. Novarini, *Hepatology,* 8 (1988) 1034.
28 W. H. Peters, F. T. Lester and H. Lubs, *Exp. Clin. Endocrinol.,* 91 (1988) 271.
29 M. Elia, P. Folmer, A. Schlatmann, A. Goren and S. Austin, *Am. J. Clin. Nutr.,* 49 (1989) 1203.
30 M. Jeevanandam, D. H. Young, L. Ramias and W. R. Schiller, *Am. J. Clin. Nutr.,* 49 (1989) 814.
31 G. A. Qureshi, A. R. Qureshi, J. Bergstrom, *J. Pharm. Biomed. Anal.,* 7 (1989) 377.
32 L. S. Eriksson, M. Olsson, and O. Björkman, *Metabolism,* 37 (1988) 1159.
33 R. Schmid, V. Schusdziarra, E. Schulte-Frohlinde, V. Maier and M. Classen, *J. Clin. Endocrinol. Metab.,* 68 (1989) 1106.