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Systematic approach to the development of plasma amino acid analysis by high-performance liquid chromatography with ultraviolet detection with precolumn derivatization using phenyl isothiocyanate☆

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ABSTRACT

A reversed-phase liquid chromatographic method for the separation of 26 phenylthiocarbamyl derivatives of amino acids in human plasma in *ca.* 35 min. is described. The method used a C_{18} column (150 × 4.6 mm I.D., 3 μm) thermostatted at 41°C, and a simple multistep linear gradient of two solvents. Solvent A was 0.05 *M* sodium acetate (pH 5.1)–acetonitrile (98:2, v/v), and solvent B was water–acetonitrile (40:60, v/v). A simple and successful approach to the optimization of the conditions for the separation of the 26 amino acid derivatives was realized. In the initial phase of development, the composition of the gradient, its timings, the column temperature, the flow-rate and the mobile phase compositions were optimized. At the end the influence of pH was studied, and this approach led to a clear resolution of the 26 amino acids. The method was validated by accuracy, precision, and recovery studies, by analyzing patient samples, and by comparing the quality control sample results with the classical ion-exchange method.

INTRODUCTION

Since the late 1980s, the classical ion-exchange chromatographic method for the analysis of amino acids (AA) has been replaced by reversed-phase high-performance liquid chromatographic (HPLC) methods that are faster, more sensitive, and inexpensive. One of the two most widely used HPLC methods involves precolumn derivatization of the amino acids with phenyl isothiocyanate (PITC). There are a few reviews on AA analysis by liquid chromatography [1,4]. The PITC method [3–14] is very common, owing to its early commercial debut, its good sensitivity (picomolar), and the advantages of PITC over *o*-phthalaldehyde.

Recent years have seen an extensive commercialization of the analysis of AAs in physiological samples by various HPLC methods. In our view, this has contributed to a scarcity of the in-depth details necessary for a quick and successful adaptation of these methods. Although there are papers dealing with the PITC method [3–14], many workers have used the "Waters Pico Tag" system (consisting of their application-specific C_{18} column, dual pumps, mobile phases, gradient program, UV detector, work-station etc.) or at least the Waters column [5,6,8–10]. Only a limited number of original systems [3,4,6,13,14] have been developed for the analysis of AAs in physiological samples using PITC, and all but one [6]

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have only partially succeeded in separating all 25 AAs. Few have described details of a successful systematic approach to the development of a method for the clear separation of all 25 AAs.

Our other objectives in the work were to (1) assemble an inexpensive, gradient HPLC–UV set-up that includes our own choice of analytical C_{18} column, (2) develop a gradient that is simple in contrast to the more elaborate methods [3,5–8,10], (3) develop a method that includes as many of the common AAs as possible, (4) verify if the assay should be performed under ion-pairing conditions, as tried by many early workers [6,11], (5) validate the method by analyzing physiological samples, and determining accuracy and precision data, and (6) reduce the analysis time per sample from 60 min or more [6,8–10].

EXPERIMENTAL

Materials

All amino acids, and the internal standard norleucine, were obtained from Sigma (St. Louis, MO, USA). Phenyl isothiocyanate was from Aldrich (Milwaukee, WI,USA). Triethylamine was from Mallinckrodt (Paris, KY, USA) and used without further purification. Sodium acetate and HPLC-grade acetonitrile were from J.T.Baker (Phillipsburg, NJ, USA). Reagent-grade water was obtained using a Milli-Q water system from Millipore (Bedford, MA, USA). The plasma ultrafiltrate was obtained using the "Centrifree" micropartition system from Amicon (Beverly, MA, USA).

Apparatus

The HPLC system consisted of two pumps (Model M45, Waters, Bedford, MA, USA), a gradient controller (Model 680, Waters), a C_{18} "Econosphere" column (150 mm × 4.6 mm I.D., 3 μm) from Alltech (Deerfield, IL, USA), a column oven (Model LC-23A) with a temperature regulator (Model LC-22A) from Bioanalytical Systems (Lafayette, IN, USA), an autosampler "Wisp" Model 710B, Waters), a UV detector (Model 655A, Hitachi, Danbury, CT, USA) with a 5-mm path-length flow-cell set at 254 nm, and an integrator plotter (Model D-2000, Hitachi). All evaporations were done using a Speed Vac Concentrator (Model SVC 100H, Savant, Hicksville, NY, USA).

Chromatographic conditions

The PITC–AA adducts were separated using a multistep linear gradient with two solvents. Solvent A was 0.050 *M* sodium acetate (pH 5.1)–acetonitrile (98:2, v/v). Solvent B was water–acetonitrile (40:60, v/v). Table I gives details of the linear gradient program (flow-rate 1.2 ml/min) used for the successful separation of the PITC derivatives.

Standard solutions

A single stock solution, containing 5 m*M* of all the 25 amino acids, was prepared in 1 m*M* hydrochloric acid made using HPLC-grade water. This standard mixture was diluted to 20, 50, 100, 200, 500, 750 and 1000 μ*M* concentrations, aliquotted into micro-centrifuge tubes and kept at −80°C. These standards are stable for *ca.* 1 year. Similarly, a working internal standard solution of norleucine of 40 μ*M* concentration was prepared, and 50 μl of the solution were added per 50 μl of ultrafiltrate prior to derivatization.

Samples

Proper sample collection, processing, and storage are of utmost importance in this work because the concentrations of many AAs are affected by these factors [1]. Immediately after collec-

TABLE I

GRADIENT PROFILE FOR THE SEPARATION OF PITC–AA ADDUCT PEAKS

Time (min)	A (%)	B(%)
0	100	0
29	52	48
31	0	100
43	0	100
44	100	0

tion, the EDTA blood was spun at *ca.* 1500 *g* for 15 min. A 500-μl aliquot of separated plasma was pipetted into an Amicon Centrifree micropartition system and ultracentrifuged at 1500 *g* for 30 min. The ultrafiltrate obtained was stored at −80°C until analysis (*ca.* 1 month).

Derivatization

A 50-μl volume of the ultrafiltrate (or the aqueous standard) and 50 μl of the 40 μM solution of the internal standard, norleucine, were pipetted into a micro-centrifuge tube, and the mixture was vacuum-dried using the Speed Vac concentrator. The procedure was repeated after adding and vortex-mixing with 10 μl of a solution of methanol–water–triethylamine (2:1:1, v/v). The AAs were derivatized using 20 μl of a solution of ethanol, water, triethylamine, and PITC (7:1:1:1, v/v). The tubes were vortex-mixed and allowed to stand at room temperature for 10 min. The tubes were vacuum-dried using the Speed Vac Concentrator. It took *ca.* 1 h to vacuum-dry 12 tubes. The residue was reconstituted in 500 μl of mobile phase A adjusted to pH 7.5, and 15 μl were injected into the HPLC system.

Method development

Cohen and Strydom successfully separated 25 amino acids in *ca.* 60 min on a 3 μm, reversed-phase C_{18} column (300 mm × 3.9 mm I.D.) [6,12] with a theoretical plate number of *ca.* 22 000. We began our separation work by choosing the Econosphere C_{18} column (150 mm × 4.6 mm I.D., 3 μm, Alltech, Deerfield, IL, USA) with a plate count of *ca.* 18 000, and a gradient system of two reagents. Reagent A was a 0.05 *M* ammonium acetate buffer of pH 6.8, and reagent B was water–acetonitrile (50:50, v/v). After a series of trials involving slight changes in mobile phase composition, flow-rate, and column temperature, and different gradient programs and timings, we tentatively optimized the chromatographic conditions. Mobile phase A was ammonium acetate (0.05 *M*, pH 6.8)–acetonitrile (98:2, v/v), and mobile phase B was water–acetonitrile (40:60, v/v). The flow-rate was 1.2 ml/min, and the gradient program close to that in Table I. At this stage of the separation work, *ca.* 70% of the 26 PITC–AA adduct peaks were clearly resolved. The real challenge of our work was reduced to the resolution of the following pairs of coeluting peaks at pH 6.8: glycine and aspargine, tyrosine and valine, ornithine and tryptophan, and leucine and isoleucine.

Attempts were made to separate the above pairs of amino acids by studying the influence of one of the variables while keeping all others constant. Although many moderate changes were noted with the different variables, the influence of pH was most dramatic and was the key to the separation of all the four pairs of peaks. When the buffer pH value was close to 7.0, glycine and asparagine coeluted, with glycine following asparagine. An increase in the temperature in this pH range only decreased the resolution of the two AAs. However, when the buffer pH was lowered to *ca.* 5.5, the order of elution reversed to asparagine first and glycine second. Further lowering of the pH below 5.5 increased the separation between the two peaks. An increase in temperature further increased this separation at low pH. Changes in gradient timings did not have any significant effect on the resolution of the two peaks.

When the buffer pH value was close to 5.2, the tyrosine and valine peaks were very clearly separated. Temperature changes did not have any significant effect on the PITC–AA adduct peaks at lower pH values. Similar improved resolution was noted between tryptophan and ornithine at lower buffer pH values and without much temperature effect. The drawbacks of the lower pH value for the work was that the late-eluting reagent peaks coeluted with leucine or isoleucine or the internal standard, norleucine. However, a slight change in the linear gradient timings moved the late-eluting reagent peaks sufficiently, and leucine and isoleucine eluted well before the reagent peak. Because a buffer of pH 5.1 better served the separation of the AAs, the mobile phase buffer was changed to a mixture of acetic acid and sodium acetate, and the column temperature was optimized to 41°C.

At least two different groups [13,14] have studied the influence of buffer pH on the resolution of

the peaks in this assay method. Both groups initially studied the separation of the PITC–AA adduct peaks using a series of buffers in the pH range 5.0–7.6. They optimized the buffer pH while keeping constant all other variables, such as analytical column temperature, gradient program, mobile phase composition, and flow-rate. This optimized buffer did not provide complete separation of all the 25 peaks, and attempts were made by the two groups to improve the resolution of the peaks by changing other variables. In contrast, we initially tried to resolve as many of the peaks by varying the gradient program, mobile phase composition, flow-rate, flow-cell dimensions, and column temperature, and at the end we optimized the buffer pH. It seems that our approach was better in that we resolved 26 peaks in *ca.* 35 min, as compared with 17 or 20 peaks by the two groups. The pH (5.1) of our buffer is different from almost all others [3–14] (pH 6.5–6.8) in this area of work, and this perhaps could partly be due to differences in the nature of the C_{18} packing materials used.

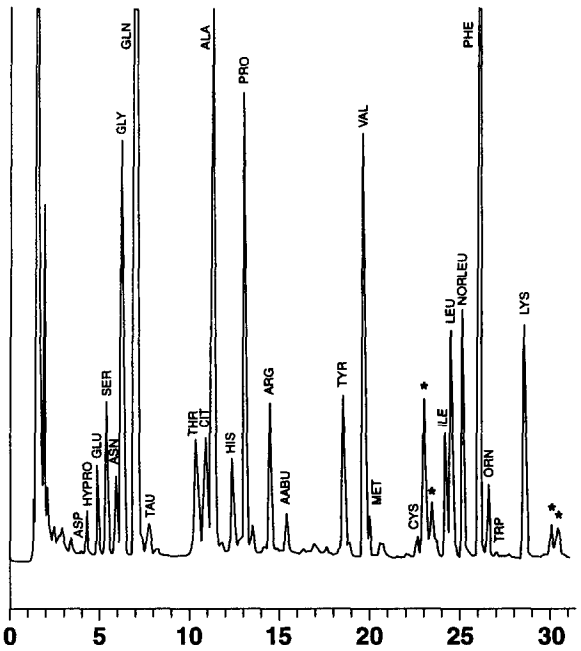

Fig. 1. HPLC separation of the PITC derivatives of 25 amino acids present in a human plasma after oral intake of "Aspartame". Reagent peaks are marked by asterisks.

RESULTS AND DISCUSSION

Fig. 1 is a chromatogram of a plasma pool ultrafiltrate carried through the derivatization process. Fig. 2 shows a chromatogram of an underivatized protein-free filtrate at 254 nm, confirming that there are no chromophores due to endogenous substances in the plasma ultrafiltrate at 254 nm. Table II gives the retention times of the PITC adducts with 35 different AAs. Table III gives details of the precision data for the method using a normal patient sample pool. The mean interassay coefficient of variation (C.V.) (n = 7) for PITC–AA adducts is 6.9%. The data compare quite well with literature reports [4,9,10]. The C.V. data for cystine and tryptophan are somewhat higher because of their well-known instability [1]. Also, the low concentrations of aspartic acid, hydroxyproline, and tryptophan exaggerate their C.V. for the method. Table IV gives details of our recovery studies for 25 amino acids (cystine omitted) by adding 20, 50, 100, and 200 μM of the standards to four differ-

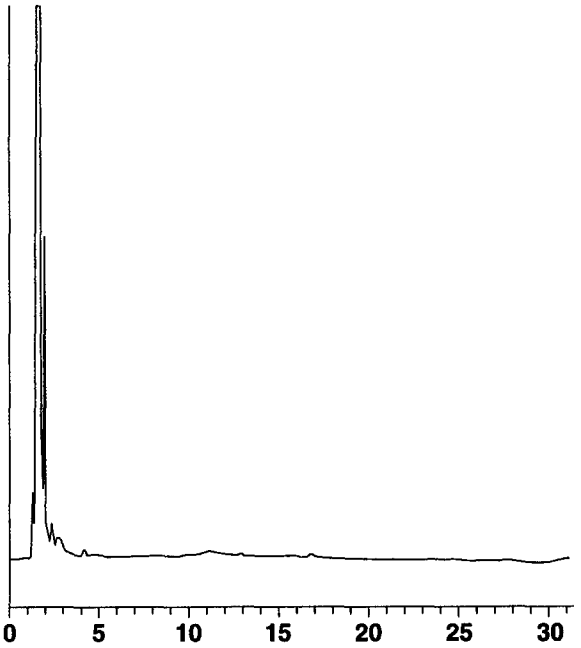

Fig. 2. Chromatogram of an underivatized protein-free ultrafiltrate of a normal human plasma (no absorption by endogenous compounds).

TABLE II

RETENTION TIMES FOR THE PITC–AA ADDUCT PEAKS

Amino acid	Relative retention time, k'
Aspartic acid (ASP)	1.79
Hydroxyproline (HPRO)	2.14
Glutamic acid (GLU)	2.45
Serine (SER)	2.94
Aspargine (ASN)	3.32
Glycine (GLY)	3.56
Glutamine (GLN)	4.14
Hydroxyserine (HSER)	4.37
Taurine (TAU)	4.51
α-Aminoadipic acid (AAAA)	6.28
Threonine (THR)	6.62
ß-Alanine (BALA)	6.71
Citrulline (CIT)	6.96
Alanine (ALA)	7.20
Methioninesulphone (METSO2)	7.61
Histidine (HIS)	7.91
Proline (PRO)	8.43
ß-Aminobutyric acid (BABA)	8.88
1-Methylhistidine (1-MH)	9.39
Arginine (ARG)	9.39
Γ-Aminobutyric acid(GABA)	9.44
Ethanolamine (EtOHNH2)	9.64
α-Aminobutyric acid (AABA)	10.14
Tyrosine (TYR)	12.44
Valine (VAL)	13.22
Methionine (MET)	13.47
Cystine (CYS)	15.31
Isoleucine (ILE)	16.45
Leucine (LEU)	16.66
Norleucine (NORLEU)	17.14
Hydroxylysine (HYLYS)	17.27
Phenylalaine (PHE)	17.79
Ornithine (ORN)	18.15
Tryptophan (TRP)	18.46
Lysine (LYS)	19.59

ent aliquots of the same normal pool that was earlier used as a quality control sample in the work. The average recovery of the amino acids was 99.9%. We found that the assay is linear up to 2 mmol. The detection limit of the assay is *ca.* 1 pmol when the signal-to-noise ratio is 5:1, and any concerns about this claim [4] appear invalid.

We validated the method by analyzing plasma samples from liver disease patients on oral hyperalimentation (Table V) to determine the absorption rate as a function of time, and samples from patients who were orally given "Aspartame" (Table VI), an experiment useful in determining its effect on the transport of AAs across the blood–brain barrier [15].

Some of the pioneering workers using the PITC method [6,11] have used ion-pairing conditions in the assay by adding triethylamine to the reagent A. The ion-pairing conditions sharpen many peaks, but many peaks coelute too [11]. The retention times of PITC derivatives often change under these conditions [11, and our unpublished results]. Without triethylamine, the retention times of peaks are highly reproducible for several assays, provided the column is in good condition. The main reasons for the poor reproduciblity of the peak retention times under ion-pairing conditions are that the second and stron-

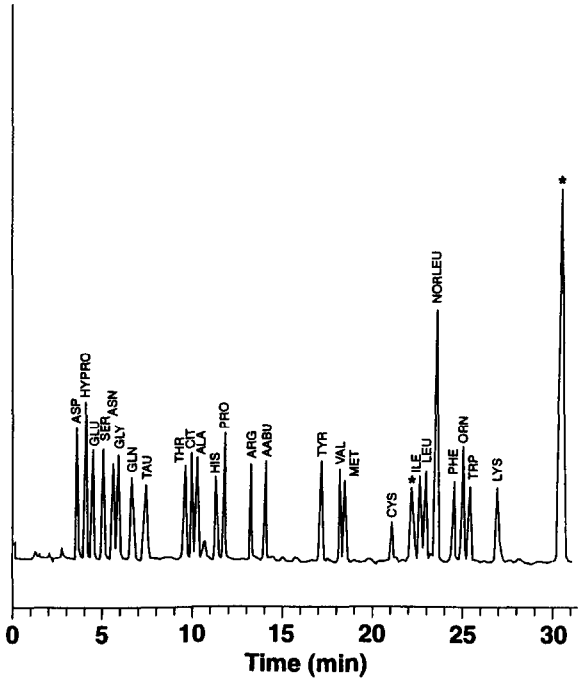

Fig. 3. HPLC separation of PITC derivatives of a standard mixture of 25 AAs (50 mmol each) and norleucine (40 mmol). Reagent peaks are marked by asterisks.

TABLE III

PRECISION DATA FOR THE PITC–AA ADDUCTS USING HUMAN PLASMA

Amino acid	Inter-assay		Intra-assay	
	Mean[a] (μM)	C.V. (%)	Mean[a] (μM)	C.V. (%)
ASP	3.1	15.6	3.3	12.6
HYPRO	7.3	13.2	7.1	7.4
GLU	21.8	4.2	22.5	4.4
SER	63.0	2.3	59.5	3.2
ASN	43.8	4.6	44.5	2.9
GLY	17O.0	5.0	184.0	3.2
GLN	608.0	1.6	592.7	2.3
TAU	31.0	2.2	26.8	3.1
THR	87.0	6.9	71.5	3.4
CIT	71.0	4.0	66.3	3.2
ALA	318.0	2.7	313.0	3.3
HIS	75.0	2.4	78.5	2.5
PRO	182.0	2.1	178.0	3.1
ARG	78.0	2.6	84.0	2.4
AABU	12.3	6.5	9.9	9.0
TYR	50.0	3.3	47.3	4.1
VAL	223.0	7.5	209.0	1.5
MET	19.2	13.9	22.8	5.3
CYS	108.0	14.4	90.0	5.0
ILE	64.0	4.1	61.0	1.9
LEU	123.0	2.8	119.0	1.7
PHE	53.0	2.8	55.0	2.1
ORN	50.3	3.5	58.0	3.1
TRP	5.1	13.0	4.6	10.2
LYS	113.0	8.2	102.0	7.3

[a] $n = 7$.

ger eluting mobile phase B washes out the ion-pairing reagent from the column, and that it takes longer to equilibrate the column with the solvent A. Even without the addition of triethylamine, the peaks are sharp and well resolved using a 5-mm path-length UV detector flow-cell and a much stronger eluting second mobile phase with 60% acetonitrile. This is in contrast to Cohen and Strydom [6] who used a mobile phase B of water–acetonitrile–methanol (40:45:15, v/v/v) to separate the PITC–AA adducts.

It is possible that the indiscrete C_{18} column that we and Ebert [11] used is not completely end-capped, and the addition of triethylamine to the mobile phase produces a higher degree of interaction between the column and the eluting PITC–AA adducts (which in turn is associated with the ion-pairing agent). Such an interaction is perhaps minimal with the Waters' amino acid C_{18} column. In any case, care should be exercised in adding triethylamine to the mobile phases in this work.

Table VII gives the mean concentrations of AAs ($n = 5$) by the HPLC method and the ion-exchange method for a normal plasma ultrafiltrate. The comparison is very good for many of

TABLE IV

MEAN PERCENTAGE RECOVERY OF AMINO ACIDS USING SPIKED PLASMA (n = 5)

Amino acid	Spiked concentration (μM)			
	20	50	100	200
ASP	92.3	92.9	90.9	87.6
HYPRO	99.8	101.3	100.9	98.0
GLU	94.8	93.2	91.4	89.1
SER	104.0	100.4	93.3	89.6
ASN	100.3	95.7	96.4	92.1
GLY	113.4	107.3	97.3	89.5
GLN	90.8	89.7	89.5	91.5
TAU	104.8	99.9	97.6	93.5
THR	95.4	94.3	94.1	87.1
CIT	106.0	103.8	97.9	89.2
ALA	105.0	100.0	88.8	88.9
HIS	101.2	104.6	103.0	101.7
PRO	100.4	86.3	95.1	93.6
ARG	109.0	99.5	98.1	95.0
TYR	101.3	101.9	99.9	97.9
VAL	93.8	88.4	88.9	85.0
MET	108.8	109.4	103.6	99.5
CYS	114.1	119.2	120.7	112.4
ILE	93.8	98.7	95.8	94.6
LEU	102.0	103.5	100.7	99.4
PHE	104.0	106.5	105.3	103.1
ORN	112.3	111.9	105.5	104.5
TRP	104.5	110.2	110.3	111.8
LYS	124.2	113.4	118.7	115.8

TABLE V

PLASMA AMINO ACIDS CONCENTRATIONS FOR A LIVER DISEASE PATIENT ON ORAL ALIMENTATION

Values are in μM.

Amino acid	Time (min)			
	0	90	180	240
ASP	6.4	12.3	15.2	13.0
HPRO	19.1	29.6	28.7	29.6
GLU	291	264	376	366
SER	133	345	351	299
ASN	68	209	191	160
GLY	299	418	392	385
GLN	374	853	833	781
TAU	32	51	52	42
THR	236	548	639	539
ALA	194	653	541	208
CIT	43	60	69	71
HIS	69	132	127	117
PRO	446	917	935	846
ARG	82	180	170	168
TYR	185	314	339	341
VAL	100	380	332	291
MET	115	211	230	242
CYS	9.6	9.6	19	14
ILE	33	202	133	109
LEU	59	313	199	146
PHE	123	255	277	274
TRP	29	38	79	65
LYS	97	462	469	391
ORN	92	144	198	203

the amino acids, but poor for the following: glycine, proline, citruline, and methionine. The first two AAs are determined by an unconventional ratio in our ion-exchange method. The lower precisions for citruline and methionine by the ion-exchange method are well known [4,5]. A similar poor precision for cystine, and the unstable nature of glutamine and asparagine, are characteristic of the PITC HPLC method.

In conclusion, our work gives greater and unique details of a systematic approach to the developmental aspect of the AA analyses by the HPLC–UV method with precolumn derivatiza-

TABLE VI

HUMAN PLASMA CONCENTRATIONS OF TYROSINE AND PHENYLALANINE FOR A PATIENT ORALLY GIVEN "ASPARTAME"

Sample	TYR (μM)	PHE (μM)
1	37	42
2	66	163
3	89	581
4	94	449
5	109	393
6	107	309
7	94	192

TABLE VII

COMPARISON OF AMINO ACID CONCENTRATIONS OF A NORMAL PLASMA OBTAINED BY THE PITC AND THE ION-EXCHANGE METHODS

Values are in μM; $n = 5$.

Amino acid	PITC method	Ion-exchange method
ASP	3.4	–[a]
HYPRO	6.2	–[a]
GLU	23.3	NM[b]
SER	62.0	69
ASN	45.5	46
GLY	171.0	275
GLN	622.4	710
TAU	59.0	62
THR	93.0	109
CIT	60.0	39
ALA	379.0	411
HIS	80.0	75
PRO	191.0	139
ARG	78.0	77
AABU	15.0	NM[b]
TYR	52.0	62
VAL	234.0	259
MET	21.0	36
CYS	88.0	71
ILE	62.0	64
LEU	121.0	141
PHE	54.0	60
ORN	45.0	68
TRP	5.0	NM[b]
LYS	156.0	176

[a] Too small to be measured.

[b] Not measured.

tion using PITC. The method is characterized by its simplicity, the use of an inexpensive and indiscrete C_{18} column, a simple gradient and a very short run-time, *ca.* 35 min per sample. We have validated the method with good precision, accuracy, and recovery data, and by analyzing patient plasma samples. We have compared our HPLC results with the classical ion-exchange method. Our method is simple and can be readily adapted for any laboratory with the basic equipment and some chromatographic skills.

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