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New high-performance liquid chromatographic method for the detection of picolinic acid in biological fluids

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Abstract

A HPLC method is described to quantify picolinic acid in milk, blood serum and tissue culture supernatant. The method requires very little sample preparation because acid precipitation allows total recovery of picolinic acid. High specificity and sensitivity were obtained using ion-pair chromatography on a C_{18} reversed-phase column with tetrabutylammonium hydrogen sulfate as ion pairing reagent. We describe the conditions for the automated testing of multiple samples and for the detection of L-tryptophan and L-kynurenine together with picolinic acid. This system will be utilized to elucidate the relationship between picolinic acid production and human disease. Furthermore, we provide the first evidence of picolinic acid in human blood serum.

Keywords: Picolinic acid; Tryptophan

1. Introduction

L-Tryptophan (TRP) catabolism along the L-kynurenine (KYN) or the serotinine pathway generates biologically active molecules that are physiologically relevant and may be implicated in several human diseases (Fig. 1) [1–9]. TRP conversion to KYN is initiated by two inducible enzymes, tryptophan dioxygenase (TDO) in the liver and indoleamine 2,3-dioxygenase (IDO) in extraepatic tissues. Under normal conditions these enzymes are silent and the catabolite levels negligible, however TDO or IDO can be readily induced to oxidize TRP to KYN by hormones or by cytokines, respectively [1]. TRP catabolites have been detected in vivo in patients treated with gamma interferon (IFN-γ) [10] or IL-2 [11] or during the rejection of transplanted tumors [12,13], and in localized compartments, such as the brain, during inflammatory conditions [14,15].

Picolinic acid (PA) is a catabolite of TRP first described in 1954 [16,17] endowed with several biological activities some of which are mediated by its ability to chelate iron and zinc [18–20]. However, the presence of picolinic acid in pathological conditions or during therapy with cytokines remains to be established. It was reported that PA has macrophage stimulatory activities [6,21,22]. Studies on the effects of exogenous PA on macrophage biology

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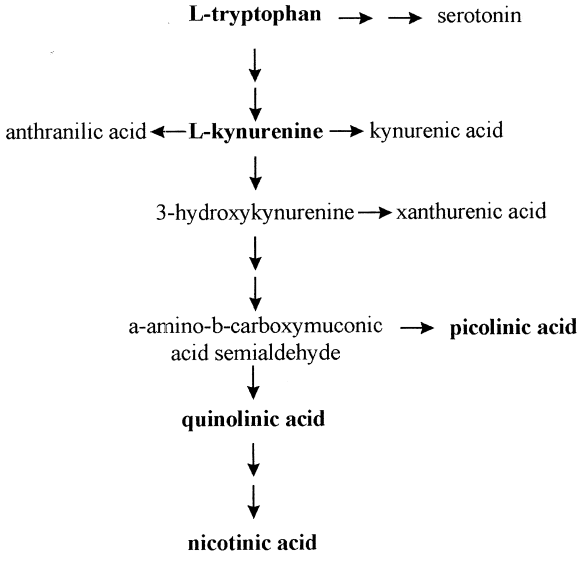

Fig. 1. Schematic representation of TRP catabolism. TRP catabolism is very complex and the figure shows only a few compounds relevant to this paper. Bold characters indicate the catabolites tested in the present study.

revealed its ability to act as co-stimulus for macrophage activation and to inhibit tumor growth in tumor-bearing mice possibly through the stimulation of the macrophage anti-tumor activity [23–30]. Moreover, PA in combination with IFN-γ inhibits retrovirus production by macrophages and triggers the expression of the inducible isoform of the nitric oxide synthase gene [29,31–33]. Finally, PA protects mice against a lethal challenge with the opportunistic pathogen *Candida albicans* and induces production of monokines [33,34]. Thus, several pieces of information indicate that PA can be used pharmacologically to modulate macrophage functions.

PA is present in dietary additives as a carrier of the divalent cation zinc or chromium and these compounds are widely distributed and consumed. Appreciation of PA biological effects and of its use as a dietary supplement spurred the interest in the possibility evaluating its levels in vivo in human specimens.

Early reports on the levels of PA in human milk relied on the determination of complexes of zinc and PA utilizing gel filtration and ion-exchange chromatography [35]. Unfortunately, these reports were quite controversial [36] and definitive information on the presence of this metabolite in human milk, serum or in other biological fluids was not established. The use of high-performance liquid chromatography (HPLC) and ion-exchange chromatography to detect PA has been proposed [37]. This method suffers from the problems that PA coelutes with NA or it remains attached to the column depending on the buffer system. Because of the growing interest in this molecule, we have developed a HPLC method to detect PA in biological fluids.

2. Experimental

2.1. Instrumentation

The HPLC separation module was a Waters Model 2690 (Milford, MA, USA) controlled by a Millennium32 data system; this module includes an autosampler with a 100-μl sample loop. In every experiment we used the SymmetryShield RP18 column (Waters), 150×3.9 mm containing 5 μm packings, and the SymmetryShield RP18 pre-column (Waters) to protect the column. TRP and methyl-DL-tryptophan (MTRP), used as an internal standard, were detected with a fluorescence detector (Waters 474 scanning fluorescence detector) at an excitation wavelength of 285 nm and an emission wavelength of 360 nm; KYN was detected by a UV detector (Waters 2487 dual λ absorbance detector) at a wavelength of 360 nm, nicotinic acid (NA), quinolinic acid (QA) and PA were detected by the same UV detector at a wavelength of 265 nm. A diode-array detector (Waters 996 photodiode array detector) was used for the identification of the peaks.

2.2. Reagents

MTRP, TRP and its catabolites, KYN, QA, PA and NA, were obtained from Sigma (St. Louis, MO, USA); methanol and perchloric acid were from Merck (Darmstadt, Germany); potassium phosphate and potassium hydroxide obtained from ICN Biomedicals (Costa Mesa, CA, USA); tetrabutylammonium hydrogensulfate (TBAHS) from Fluka (Buchs, Switzerland). All chemicals were HPLC grade. Sera from normal donors and patients were

collected in the Gastroenterology Department, Molinette Hospital, Turin, Italy.

2.3. Cell culture

The human hepatoma cell lines Hep3B and human monocytic cell lines THP-1 were obtained from American Type Culture Collection. The Hep3B was cultured in Dulbecco's modified Eagle's medium (ICN Biomedicals, Aurora, OH, USA) supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1× non-essential amino acids, 2 m*M* L-glutamine and 1 m*M* sodium pyruvate (all from Hyclone Labs., Logan, UT, USA). The THP-1 was cultured in RPMI 1640 medium (ICN Biomedicals) supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 m*M* L-glutamine (all from Hyclone Labs.). The cells were seeded in 75 cm^2 flasks at a cell density of $2 \cdot 10^5$ cell/ml for the Hep3B and $5 \cdot 10^5$ cell/ml for THP-1 and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Aliquots of the tissue culture supernatants were harvested at different time points and processed for HPLC analysis.

2.4. Sample preparation

Excellent results were obtained when we used acid precipitation to dissociate the putative PA–protein complexes and to extract the samples. Every sample was spiked with MTRP before the acid precipitation. Blood serum or tissue culture supernatant were adjusted to a final concentration of cold 2% perchloric acid (PCA), kept on ice for 30 min, centrifuged 5 min at 13 000 *g* and the pellet was discarded. The PCA-soluble fraction was neutralized by adjusting to 0.4 *M* potassium hydroxide, the clear supernatant was filtered through a 0.45-μm filter (13 mm GHP 0.45 μm Minispike from Waters) and analyzed by HPLC. A 100% recovery MTRP, TRP, PA and other catabolites was achieved under these conditions. Milk samples had to be ultracentrifuged at 100 000 *g* for 20 min before the PCA precipitation to remove the cell pellet and the upper lipid phase. The clear liquid phase was then precipitated with PCA and processed as described above.

2.5. Identification of tryptophan catabolites

The identification of tryptophan catabolites was achieved by comparing the retention times and spectral data (obtained by diode-array detection) with the standard and by spiking the samples with PA, KYN and TRP.

2.6. Calculation and reproducibility

The unknown metabolite concentration was determined from the regression equation relating the peak-area to their concentration. Correlation and regression were analyzed by the least-squares method. The reproducibility of the system was controlled by MTRP counts. Considering the total standard deviation of the method, variation of MTRP$<$5% was tolerated.

As shown in Table 1, the precision of the method was estimated by measuring the within-day and between-day reproducibility of the analytes at four different concentration. The mean values for within-day reproducibilities were 2.1% for TRP, 1.3% for KYN and 0.8% for PA and the mean values for between-day reproducibilities were 2.7% for TRP, 1.4% for KYN and 1.2% for PA. The specimens were prepared on 1 day and were frozen in aliquots of 100 μl at −20°C.

3. Results and discussion

We were interested in measuring PA in blood serum, milk serum and tissue culture supernatant. PA is difficult to handle because it aggregates, binds to the column matrix and forms complexes with proteins. In fact, the preparation of samples using extraction with organic solvents (ethyl acetate, benzene, pyridine, phenol), with a C_{18} or with a ion-exchange cartridge could not be used because the recovery of PA was less than 30% and we had to use acid precipitation to dissociate PA–protein aggregates. Furthermore, preliminary attempts to utilize reversed-phase or ion-exchange chromatography for PA separation were unsuccessful because PA eluted with wide peaks, remained attached to the column or separated in multiple peaks (data not shown). We decided to try ion-pair chromatography to separate

Table 1
Reproducibility of the HPLC method

	TRP		KYN		PA	
	Concentration (μM)	RSD[a] (%)	Concentration (μM)	RSD[a] (%)	Concentration (μM)	RSD[a] (%)
Within-day (n=5)	10	2.7	5	1.6	5	1.2
	20	1.9	10	1.5	10	0.8
	40	2.5	20	1.3	20	0.7
	80	1.6	40	1.3	40	0.5
	100	1.8	80	0.9	80	0.9
	Mean	2.1		1.3		0.8
Between-day (n=10)	10	2.8	5	1.7	5	1.5
	20	3.1	10	1.2	10	1.2
	40	2.5	20	1.5	20	1.2
	80	2.8	40	1.2	40	0.9
	100	2.1	80	1.4	80	1.3
	Mean	2.7		1.4		1.2

[a] RSD=Relative standard deviation.

PA employing a C_{18} reversed-phase column, 1 m*M* TBAHS as ion-pairing reagent and phosphate buffer. We tested the binding and elution of PA at different pH, methanol and TBAHS concentrations. The effect of increasing concentrations of methanol and of different pH on the elution of PA is shown in Fig. 2. We found that the retention time of PA could be increased from 2.3 to 3.7 min by decreasing the methanol concentration from 30% to 2%. The decrease of the pH of the phosphate buffer from 8 to 6 delayed the elution of PA from 3.7 to 4.8 min but also increased the width of the peak. On the basis of these results we have chosen an isocratic elution with a mobile phase composed by 30 m*M* phosphate buffer, pH 8, 2% methanol and 1 m*M* TBAHS because PA eluted with a sharp and symmetric peak with a retention time of 3.8 min. These conditions will be used for the following experiments.

We tested whether the above condition could resolve a mixture of tryptophan catabolites containing TRP, KYN, PA, QA, NA and MTRP (Fig. 3A). We found that we could separate all these compounds which eluted with retention times of 1.9 min for NA, 2.4 min for QA, 3.7 min for PA, 4.9 min for KYN, 11.2 min for TRP and 13.8 min for MTRP. Utilizing UV absorption at 360 nm or fluorescence at an emission wavelength of 360 nm following an excitation wavelength of 285 nm we could detect selectively KYN (Fig. 3B) or TRP and MTRP (Fig. 3C), respectively. It is important to note that this method clearly resolves PA and NA, which are isomers and differ only in the position of the carboxyl group on the pyridinic ring. These results demonstrate that PA and other TRP catabolites can be resolved with ion-pair chromatography in a 15-min run.

We tested whether the above conditions could be used to measure PA in biological fluids. Fig. 4 shows a representative elution profile of normal human blood serum before and after the addition of 20 μM PA. PA eluted with a specific retention time not shared by other serum molecules most of which eluted in less than 3 min. TRP and KYN could also be quantified in the same run because the peaks not disturbed by other components. QA and NA could not be measured under these conditions because they co-eluted with other serum molecules. Similar results were obtained when we used other biological material such as cow or human milk or the supernatants of human cells (THP1 and Hep3B) cultured for 24 or 72 h (Fig. 5).

Next, we established the conditions to regenerate the column for automated testing of multiple samples (Table 2). After 15 min of isocratic elution, the column was washed with a mobile phase composed of 50% methanol and 30 m*M* phosphate buffer, pH 8

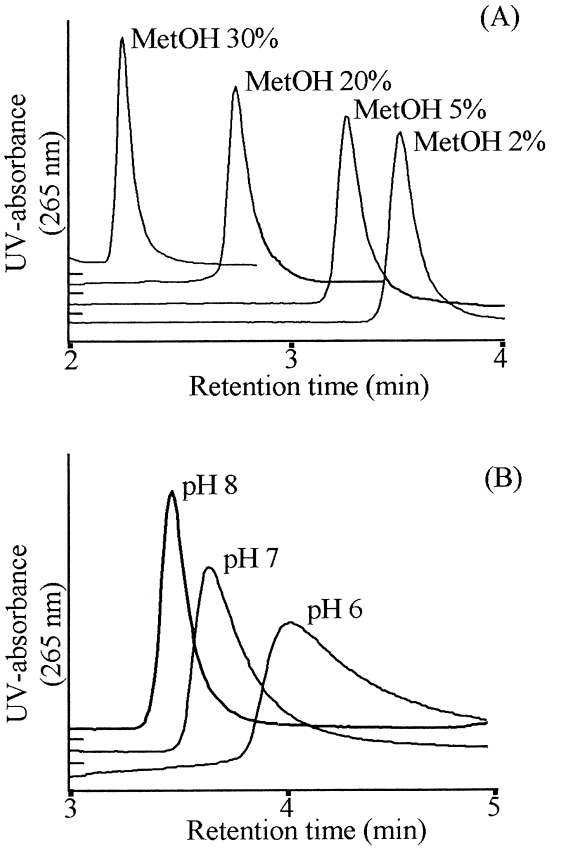

Fig. 2. Effect of methanol concentration and pH on PA elution. A 10-μl volume of 20 μ*M* PA solution was injected and separated on a C_{18} column equilibrated with the buffer detailed below. (A) Mobile phases containing 1 m*M* TBAHS, 30 m*M* phosphate buffer, pH 8 and methanol at concentrations of 30%, 10%, and 2%; (B) mobile phases containing 1 m*M* TBAHS, 2% methanol and 30 m*M* phosphate buffer at pH 6 in, 7 and 8. Detection: UV at 265 nm.

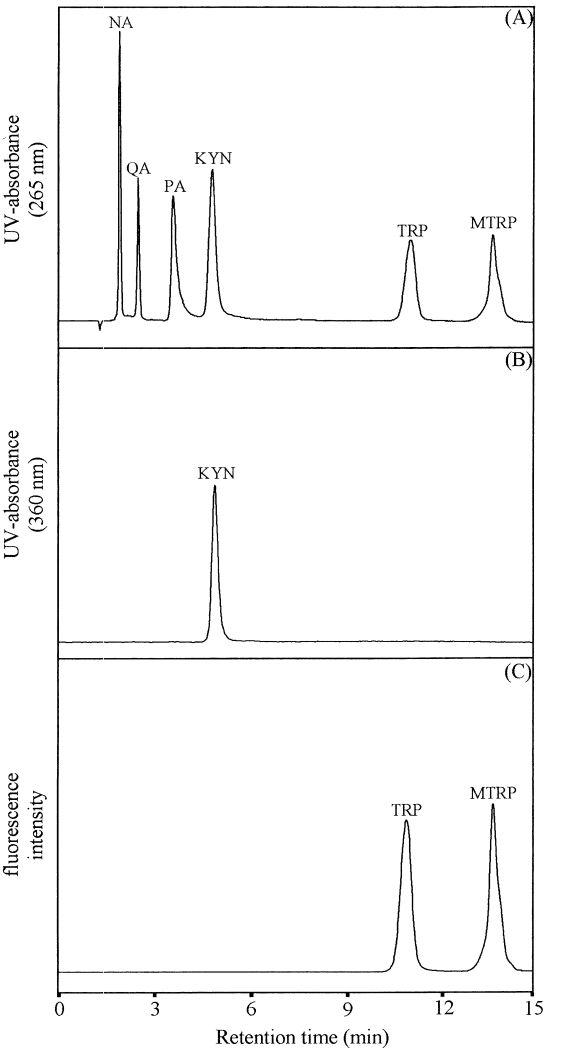

Fig. 3. Separation of TRP metabolites and the internal standard methyl-DL-tryptophan. The standard mixture contained: 40 μ*M* L-tryptophan (TRP), 20 μ*M* L-kynurenin (KYN), 20 μ*M* picolinic acid (PA), 20 μ*M* quinolinic acid (QA), 20 μ*M* nicotinic acid (NA) and 40 μ*M* methyl-DL-tryptophan (MTRP). A 10-μl volume of the mixture was injected onto a C_{18} column equilibrated in a running buffer composed of 2% methanol, 1 m*M* TBAHS, 30 m*M* phosphate buffer, pH 8 and eluted isocratically for 15 min. (A) Chromatogram obtained by UV absorption at 265 nm. (B) Chromatogram obtained by UV adsorption at 360 nm. (C) Chromatogram obtained by fluorescence detection at an excitation wavelength of 285 nm and an emission wavelength of 360 nm.

at an initial flow of 0.5 ml/min to reduce the surge in pressure due to the higher methanol concentration. The transitions from one condition to the next always followed a linear gradient profile from here on. The flow-rate was slowly increased to 0.8 ml/min before switching to the equilibration buffer containing 10-times more TBAHS than the running buffer to shorten the equilibration time. After a short equilibration in the running buffer the column was ready for the next sample. Overall, the total time needed to process a sample was 60 min. The perfect overlapping of two chromatograms of the same serum spiked with standards obtained before and after the analysis of 30 unrelated serum samples, demonstrates that the system is reproducible and that

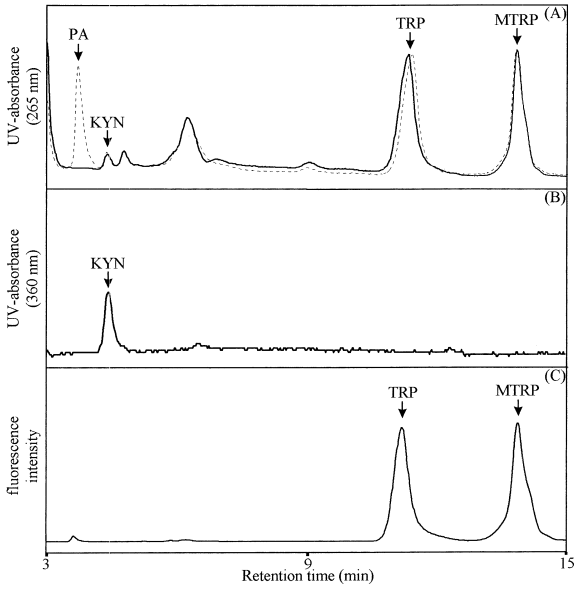

Fig. 4. Representative chromatograms of human blood serum from a normal donor. (A) Chromatogram of the serum either not spiked (continuous line) or spiked with 20 μ*M* PA (dotted line) obtained by UV absorption at 265 nm. (B) Chromatogram of not spiked serum analyzed by UV adsorption at 360 nm. (C) Chromatogram of not spiked serum analyzed by fluorescence detection at an excitation wavelength of 285 nm and an emission wavelength of 360 nm.

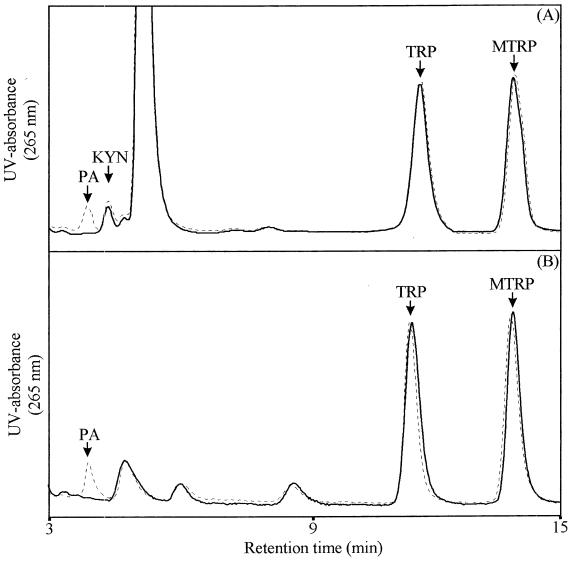

Fig. 5. Representative chromatograms of human milk and supernatant of human cells. (A) Chromatogram of the supernatant of THP1 monocytic cells either not spiked (continuous line) or spiked with 10 μ*M* PA (dotted line) obtained by UV absorption at 265 nm. (B) Chromatogram of human milk either not spiked (continuous line) or spiked with 20 μ*M* PA (dotted line) obtained by UV absorption at 265 nm.

the washing is efficient (data not shown). We were unable to find other conditions allowing such a reproducibility among different runs requiring a shorter column regeneration.

In order for an analytical technique to perform quantitatively, it needs to be both linear and reproducible. The reproducibility was evaluated by analyzing 10 replicates of 20 μ*M* PA as a representative sample. The relative standard deviation (RSD) was 1.2% of the mean value and the correlation coefficient was 0.99. The linearity and detection limits were determined by analysis of standard solutions from 0.2 μ*M* to 4 m*M* for PA, from 0.09 μ*M* to 4 m*M* for KYN and from 0.05 μ*M* to 1 m*M* for TRP and MTRP. The standards were acidified and treated as the samples before the analysis. The signal intensities were proportional to concentrations in these ranges. The detection limits, at a signal-to-noise ratio of 3 with an injection volume of 10 μl, are presented in Table 3. The sensitivity of TRP and KYN are comparable to those reported with other methods of detection [8,9]. The detection limit of 0.2 μ*M* for PA is in the range of the detection limit of KYN (about twice as high) and it is 10-times more sensitive than previously reported separations [37]. It may be possible that derivatization protocols or other

Table 2
Multiple run set-up

Time (min)	Flow (ml/min)	% RB[a]	% WB[b]	% EB[c]
1	1	100		
15	1	100		
16	0.5	100		
21	0.5		100	
33	0.8		100	
36	1			100
46	1			100
47	1	100		
60	1	100		

[a] RB=Running buffer: 2% methanol, 1 m*M* TBAHS, 30 m*M* phosphate buffer, pH 8.

[b] WB=Washing buffer: 50% methanol, 30 m*M* phosphate buffer, pH 8.

[c] EB=Equilibrating buffer: 2% methanol, 10 m*M* TBAHS, 30 m*M* phosphate buffer, pH 8.

Table 3
Fluorescence (FL) and UV detection limits

Compound	Detection limit[a]	
PA	0.2 μ*M*	UV (265)[b]
KYN	0.1 μ*M*	UV (265)
	0.09 μ*M*	UV (360)[c]
TRP	0.5 μ*M*	UV (265)
	0.02 μ*M*	FL (285/360)[d]

[a] Detection limits=signal-to-noise ratio of 3 and injection volume of 10 μl.
[b] UV (265)=UV absorption at a wavelength of 265 nm.
[c] UV (360)=UV absorption at a wavelength of 360 nm.
[d] FL (285/360)=Fluorescence intensity at an excitation wavelength of 285 nm and an emission wavelength of 360 nm.

detection system will increase the sensitivity of the method. Relatively high concentrations of PA seem to be required to achieve a biological response at least in macrophages and there are no indications that tryptophan metabolites are biologically relevant at n*M* concentrations, although the latter cannot be excluded.

Furthermore, experiments were performed in which adult wistar rats were given 1 mg/ml PA in drinking water and tested for PA in blood serum. This regimen caused the appearance of a peak of PA in the blood serum in concentration ranging from 30 to 50 μ*M*. These results validate the detection system clearly identifying the peak detected in the serum as PA.

In an attempt to determine whether PA was produced in vivo, we screened sera from normal donors or patients affected by different diseases. We found PA below the detection limit in more than 90 sera from normal donors. These results are in agreement with the current notion that TRP catabolism is an inducible process mainly associated with a pathological situation [1,2,7]. However, when we tested a batch of 20 sera from patients with liver cirrhosis we found that two of them contained relatively high PA levels (18 μ*M* and 41 μ*M*, respectively). The elution profile of the serum containing 18 μ*M* PA before and after spiking with 20 μ*M* PA is shown in Fig. 6. The overlapping of the endogenous and exogenous PA peak and the analysis of the spectral data of sample peak indicate the presence of this molecule in the patients' serum.

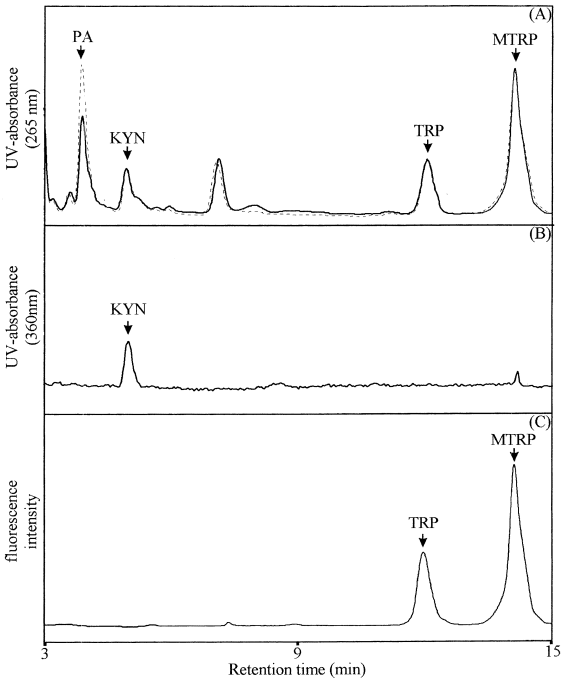

Fig. 6. Chromatograms of human serum from a patient with liver cirrhosis. (A) Chromatogram of serum from a patient either not spiked (continuous line) or spiked with 20 μ*M* PA (dotted line) obtained by UV absorption at 265 nm. (B) Chromatogram of not spiked serum obtained by UV adsorption at 360 nm. (C) Chromatogram of not spiked serum obtained by fluorescence detection at the excitation wavelength of 285 nm and the emission wavelength of 360 nm.

Similar results were obtained in three independent runs of the same sample. Moreover, we changed the experimental conditions by using a XTerra RP18 (Waters) 150×3.9 mm column containing 5 μm packings and a mobile phase composed by 0.5 m*M* borate buffer, pH 9, 2% methanol and 1 m*M* TBAHS. Under these conditions the elution times changed substantially but the pattern of results and levels of PA detected remained similar (data not shown).

These data provide the first evidence of the presence of PA in human serum. Even if relatively high PA levels were found in patients with liver cirrhosis, it is premature to suggest any correlation between the PA and clinical manifestation. We could speculate that liver degeneration caused the deregulation in TRP catabolism in hepatocytes that may be shifted toward PA production. Alternatively, the

kuppfer cells responding to signals originating from the suffering liver cells may be activated to produce PA. We cannot exclude that the PA originated from other tissues in response to liver failure, generalized toxic status of the patient or drug intake.

4. Conclusions

In conclusion, we have developed a new method for PA detection in biological fluids that has proven its validity in detecting for the first time the presence of this metabolite in the patients' serum. This method is reliable and reproducible and it has been successfully applied to the automated analysis of multiple samples. It is now possible to address issues of pathophysiology of PA production and to gain insights on the nature of the PA producing cell.

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