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Optimization of conditions for the simultaneous separation of ten tryptophan metabolites using reversed-phase high-performance liquid chromatography

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ABSTRACT

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the separation of tryptophan and ten metabolites of tryptophan pyrrolase pathway has been developed by sequential optimization of mobile phase, by adjusting the pH, the concentration of triethylamine and the gradient elution The baseline resolution of the compounds, by this optimized procedure, is obtained with an analysis time, including the re-equilibration period, of less than 30 min We believe this is the first RP-HPLC method that can separate tryptophan and ten of its metabolites in a single chromatographic run.

INTRODUCTION

There are at least two major pathways for tryptophan (TRP) metabolism. The first pathway leads to the formation of serotonin by hydroxylation followed by decarboxylation of TRP. The second pathway, the pyrrolase pathway, results in formation of a series of metabolites following cleavage of the indole ring (Fig. 1).

At least two of the metabolites of TRP pyrrolase pathway – quinolinic acid and kynurenic acid – have been reported to play important roles in excitatory neurotransmission, neurotoxicity and epilepsy [1–4] In addition kynurenine has

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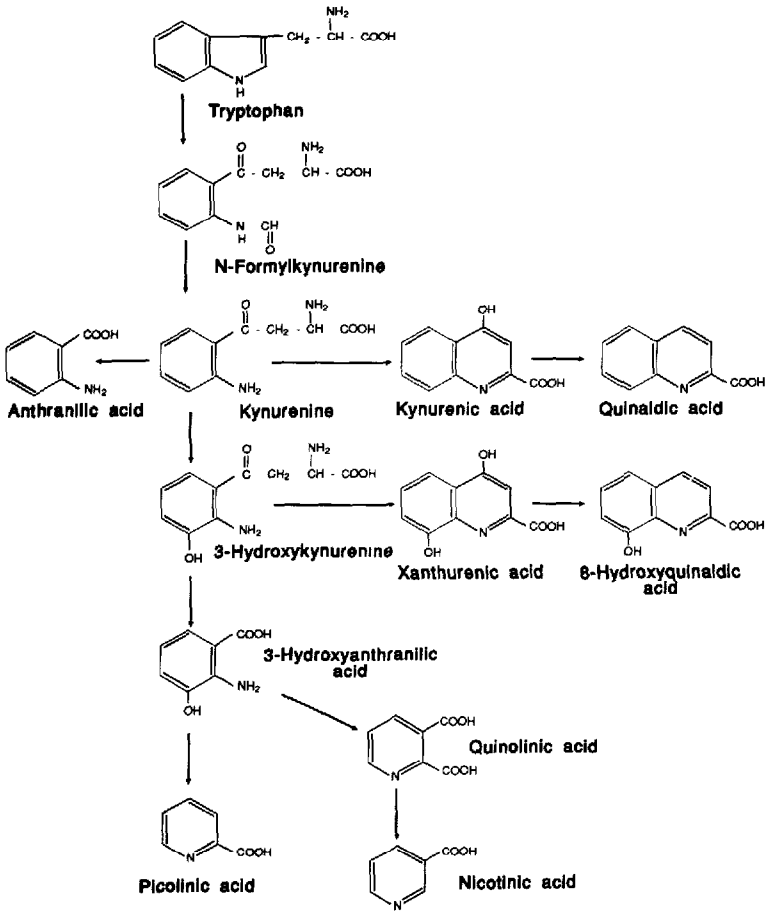

Fig 1. Pathway of tryptophan metabolism

been shown to act as an excitotoxin in an *in vitro* study [5]. Studies into the role of other TRP metabolites in the pathogenesis of human neurological disorders are limited by the technology available to separate and quantitate these compounds in biological samples. The reported analytical methods include gas chromatography with electron-capture detector [6], gas chromatography–mass spectrometry (GC–MS) [7–9] and reversed-phase high-performance liquid chromatography (HPLC) [10–14] used alone or in combination to measure a single (quinolinic acid and kynurenic acid [7–11]) or only few metabolites of TRP pyrrolase pathway [6,12–14]. Quinolinic acid, a TRP metabolite with a major role in central nervous system function [15,16], has generally not been included with other metabolites in previous analytical procedures; therefore, there is a need for a new sensitive method that can separate and measure many metabolites including quinolinic acid in a single sample.

All of the TRP metabolites are ionogenic compounds, containing at least one carboxylic and one amine group (primary, secondary or tertiary); some of the compounds have up to two hydroxyl groups attached to the ring structure. Both the polar functional group(s) and non-polar ring structure contribute to the compound's distinct physicochemical properties (polarity, K_a, hydrocarbonaceous surface area) which determine the retention mechanism when chromatographed by HPLC [17,18].

In HPLC lipophilic counter ions are commonly added to the aqueous mobile phase to increase retention of ionized solutes. This technique, called ion-pair chromatography, has been successfully applied to separation of ionizable compounds [19–23]. The pH and the concentration of counter ion (*i.e.* ion-pair reagent, ion-interaction reagent, surface-active ion) in the eluent are two most powerful factors that can be used in ion-pair chromatography to control the retention and selectivity of ionic compounds [24–28]. We included triethylamine (TEA) in the mobile phase to facilitate the separation of TRP metabolites by HPLC in this study.

In this paper we present the first HPLC method that can separate ten metabolites of TRP in the pyrrolase pathway This was accomplished by sequential optimization of the mobile phase pH, the concentration of TEA and by the use of gradient elution.

EXPERIMENTAL

Apparatus

The HPLC apparatus consisted of a WISP 710B autosampler, two M6000A solvent delivery systems, an M440 absorbance detector, an M730 data module and an M720 system controller. Chromatographic separations were performed on a Nova-Pak C_{18} steel column (150 mm × 3.9 mm I.D., 4 μm). A Guard-Pak cartridge holder with μBondapak C_{18} insert was connected between autosampler and column. The apparatus and materials were obtained from Waters (Milford, MA, U.S.A.).

Chromatographic conditions

The mobile phase consisted of a binary linear gradient of solution A (20 m*M* phosphoric acid, 20 m*M* acetic acid, 0–40 m*M* TEA adjusted to desired pH with 2 *M* sodium hydroxide) and solution B (acetonitrile). Except for conditions mentioned in the section on optimization of the gradient, the gradient program was as follows: starting from 100% A to 20% A–80% B within 20 min (0.8 ml/min flow-rate), then the gradient was reversed over a 2-min period to 100% A. The column was re-equilibrated with five column volumes of solution A before injection of the next sample. The column was equilibrated with solution A for at least twenty column volumes for each mobile phase change. The column dead time, t_0 (1.53 min at 0.8 ml/min), was determined by injecting a uracil solution.

The detection wavelength was set at 254 nm and sensitivity at 0.1 or 0.2 a.u.f.s. A 10-μl volume of a standard mixture was injected in duplicate and all chromatographic runs were performed at 22–24°C.

Calculation of b value in gradient elution

The b value was calculated using the following equation [29]: $b = \emptyset S t_0$ where, $\emptyset$ = rate of change of solution B with time (gradient rate or gradient steepness) expressed as volume fraction per min, S = solvent strength parameter and t_0 = column dead time in min. S is dependent upon separation parameters; its approximate value for acetonitrile is taken as 3.1 [29].

Reagents

All standards (trytophan and its ten metabolites) and 2-pyridylacetic acid HCl (Table I) were purchased from Aldrich (Milwaukee, WI, U.S.A.) or Sigma (St. Louis, MO, U.S.A.). Concentrated stock solution of standards (1 mg/ml) were prepared in deionized water or 10% methanol and stored at −20°C. Mixtures of multiple standards (50 μg/ml) were prepared in mobile phase solution A and stored at −20°C. All other chemicals were of reagent grade obtained from Mallinckrodt (Paris, KY, U.S.A.) or Aldrich. Acetonitrile (HPLC grade) was purchased from Baker (Phillipsburg, NJ, U.S.A.). Water was purified by Millipore Milli-Q system (Waters).

RESULTS AND DISCUSSION

Our goal was to develop an RP-HPLC method that will separate TRP and ten

TABLE I

ABBREVIATIONS USED FOR DIFFERENT TRYPTOPHAN METABOLITES

Peak No	Compound	Abbreviation
1	Quinolinic acid	QA
2	3-Hydroxykynurenine	HK
3	3-Hydroxyanthranilic acid	HA
4	Picolinic acid	PA
5	Nicotinic acid	NA
6	Kynurenine	KN
7	2-Pyridylacetic acid	PAC[a]
8	Anthranilic acid	AA
9	Tryptophan	TRP
10	Xanthurenic acid	XA
11	Kynurenic acid	KA
12	Quinaldic acid	QN

[a] To be used as an internal standard

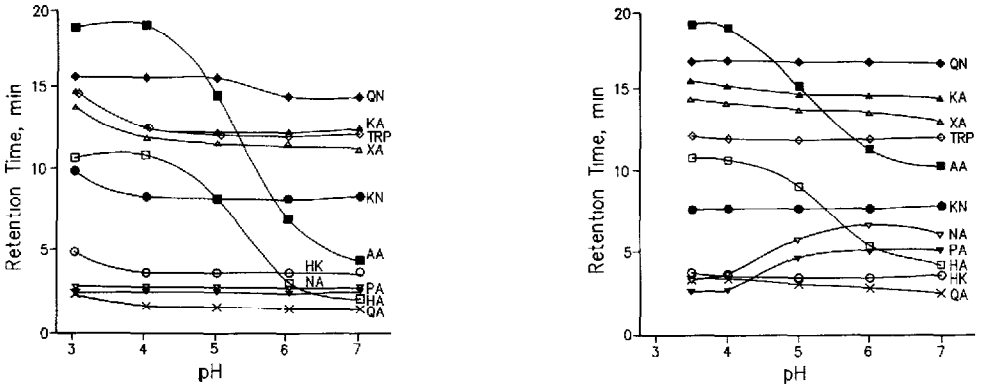

Fig. 2 Effect of mobile phase pH on retention (without triethylamine added). Chromatographic conditions solution A (20 m*M* acetic acid, 20 m*M* phosphoric acid, pH adjusted with 2 *M* sodium hydroxide solution), solution B (100% acetonitrile), gradient of B from 0 to 20% within 20 min; flow-rate, 0 8 ml/min. See Table I for the abbreviations of different metabolites.

Fig 3 Effect of mobile phase pH on retention (with triethylamine added) Chromatographic conditions were the same as described in legend to Fig. 2 except 20 m*M* triethylamine was added to the solution A See Table I for the abbreviations of different metabolites

of its metabolites in the pyrrolase pathway with resolution equal to or greater than 1.5 for each near-by peak pair and a run time less than 30 min. Acetonitrile was chosen as the organic mobile phase because higher efficiency can be obtained with acetonitrile at a lower pressure when mixed with an aqueous mobile phase. Since mobile phase pH is the major factor that affects the ionization of compounds under study and determines solute separation and selectivity [19–21,28], it was chosen as the first parameter to be optimized.

Optimization of mobile phase pH

The retention of these eleven compounds at different pH values of solution A without TEA added is shown in Fig. 2. Three of the metabolites – quinolinic acid (QA), nicotinic acid (NA) and picolinic acid (PA) – eluted rapidly (less than two column dead times about 3 min) between pH 3 and 7. Kynurenic acid (KA) and TRP were always coeluted in the above pH range. Due to the instability of silica-based columns to pH below 2.5 and above 7.5, only the pH range 3–7 was tested.

In order to increase the retention of fast eluting compounds, TEA as added to the aqueous mobile phase as ion-pair reagent. TEA has been used successfully as an ion-pair reagent for the separation of nucleotides by reversed-phase column chromatography [30]. It does not have a deleterious effect on the stationary phase like that of tetrabutylammonium; in addition it is very water-soluble and has a low absorbance in the UV range [30]. TEA is a small ion-pair reagent having a short hydrophobic alkyl chain, therefore, it is not only rapidly (re)equilibrated in

the column but also easily eliminated from the column [19]. This makes it practical to be used in gradient elution [19]

The retention of eleven compounds at different pH values of solution A containing 20 m*M* TEA is shown in Fig. 3. The lowest pH used was 3.5; solution A of pH 3.0 could not be obtained with the buffer concentration used. Only at about pH 7.0 were the eleven compounds well resolved and their retention times stable with minor pH changes. But the retention time of QA remained less than 3 min. At pH 7.0, carboxylic groups are completely ionized as anions and interacted with triethylammonium ions (pK_a = 10.7 [31]).

The peak shapes of PA and quinaldic acid (QN) were also affected by pH change (data not shown). PA and QN peaks at mobile phase pH 5.0 were broad and tailing; in addition, the QN peak also presented itself as a doublet. None of these peak behaviors were affected by the presence of TEA. At pH 7.0, however, the above problems were eliminated.

Optimization of TEA concentration

Mobile phase of pH 7.0 was chosen to study the effect of TEA concentration on the retention of eleven compounds (Fig. 4). The retention of three compounds – TRP, kynurenine (KN) and 3-hydroxykynurenine (HK) – were only slightly affected within a TEA concentration range of 0–40 m*M*. The remaining compounds showed increased retention with increasing TEA concentration.

These phenomena indicated that TEA is functioning as an ion-pair reagent. At pH 7.0 TRP, KN and HK are neutral zwitterions and, therefore, are not affected by increases in triethylammonium ion. The other eight compounds are present as anions which can interact with thriethylammonium cation, and thus their retentions are increased with increasing counter-ion concentration. The best condition

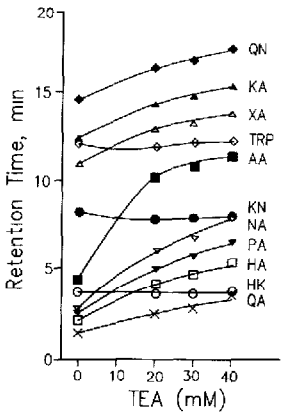

Fig 4. Effect of concentration of triethylamine on retention at mobile phase pH of 7 0 Other chromatographic conditions were the same as described in legend to Fig 2 See Table I for the abbreviations of different metabolites

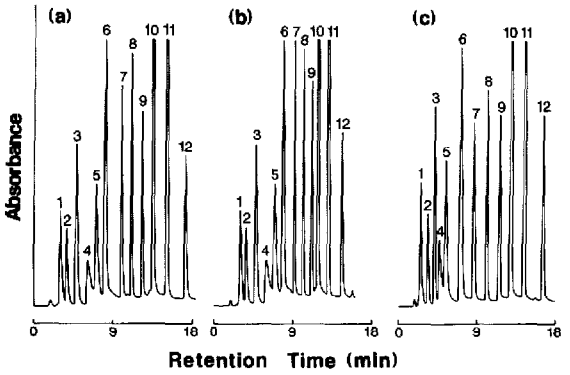

Fig. 5. Chromatograms after sequential optimization of mobile phase pH, TEA concentration and gradient parameters for the best separation of TRP and ten of its metabolites Chromatographic conditions: solution A (20 m*M* acetic acid, 20 m*M* phosphoric acid, 30 m*M* TEA, pH 7 0 adjusted with 2 *M* sodium hydroxide solution), solution B (100% acetonitrile) Gradient conditions are as described in Table II (a) condition 1; (b) condition 4, (c) condition 8 See Table I for peak identification of each compound, a.u f.s. = 0.1, 500 ng of each compound was injected

TABLE II

GRADIENT CONDITIONS AND THEIR EFFECTS ON RESOLUTION, DETECTION SENSITIVITY AND ANALYSIS TIME

Mobile phase solution A (20 m*M* acetic acid, 20 m*M* phosphoric acid, 30 m*M* TEA, pH 7 0, adjusted with 2 *M* sodium hydroxide solution), solution B (100% acetonitrile)

No	Gradient rate (% solution B/min)	Flow-rate (ml/min)	Initial solution (% B)	Retention time[a] (min)	Relative sensitivity[b]	*b*[c]	*R*[d]	Critical pairs(s)
1	1	0 8	0	3 08–17.10	1 0	0 05	1.0	PA/NA
2	1	1 2	0	1 55–11.79	0.91	0 03	0 6	PA/NA
3	1	0 6	0	3.95–20 11	1 0	0 06	1 0	PA/NA
4	1 5	0.8	0	3.03–14 51	1.0	0 07	1 0	PA/NA
5	2 0	0 8	0	3 08–13 15	1 0	0 1	1.0	PA/NA
							1 3	TRP/XA
6	1.5	0.6	0	3 96–17 30	1 04	0.09	1 0	PA/NA
							1 3	TRP/XA
7	2.0	0 6	0	3.98–15.76	1 02	0 12	1 0	PA/NA
							0 9	TRP/XA
8	1	0 8	1	2.55–16 38	1.39	0 05	1.1	PA/NA
9	1	0.8	2	2 30–15 39	1 54	0.05	0 8	PA/NA

[a] Retention time of peaks the range of retention times between first and last eluted peaks.

[b] Relative sensitivity relative peak height of first eluted peak to that of condition 1.

[c] See experimental section for the details of calculation

[d] Resolution of critical pairs ($R < 1$ 5), compared to Table 2 1 of ref. 32.

for resolution of all eleven components while keeping the earliest eluting peak (QA) near 3 min was 30 m*M* TEA. A chromatogram obtained with 30 m*M* TEA shows all compounds well resolved (resolution equal to or greater than 1.5) except for the PA–NA pair (Fig. 5a).

Optimization of gradient elution

The gradient parameters (flow-rate, gradient rate and initial gradient concentration) were evaluated to further optimize the separation. Optimization of any of these parameters is often at the expense of others [29]. The effects of changing the parameters on resolution of critical pairs, retention of first and last peaks, and detection sensitivity are shown in Table II. Resolution was determined by comparison to Snyder's resolution figures [32] while detection sensitivity was determined by the relative peak height of the first peak to that of condition 1. The condition 1 is the same as described under Experimental except the concentration of TEA was 30 m*M* with mobile phase pH at 7.0.

Gradient condition 8 of Table II was found to be the best in terms of resolution, detection sensitivity and analysis time; however, the retention time of the first eluting peak was less than $2t_0$. If peak elutions of equal to or greater than $2t_0$ were to be achieved, conditions 1 and 4 will be the best choices. However, the sensitivity of the early eluting peaks was lower than that of condition 8. Chromatograms of these optimal conditions are shown in Fig. 5. All compounds except PA were well resolved (R equal to or greater than 1.5), and retention time of the last peak was within 18 min. The b values for these optimal conditions were between 0.05 and 0.1, which is in agreement with ref. 33 for particle size less than 10 μm of column packing.

CONCLUSION

There have been many excellent reports of the separation and measurement of multiple TRP metabolites [12–14]; however, these investigations have generally not included QA, a metabolite of major interest in neurochemistry. For example, Krustulovic *et al.* [13] have achieved separation of TRP, KN, HK, anthranilic acid (AA) and 3-hydroxyanthranilic acid (HA), whereas Morita *et al.* [14] have concentrated on separating TRP, KN, HK, AA, HA, KA and xanthurenic acid.

This HPLC method for the separation of eleven compounds (TRP and its ten metabolites in the pyrrolase pathway) has been developed by sequential optimization of mobile phase pH, concentration of TEA and gradient elution. The resolution obtained is very good and has an analysis time that is practical. To our best knowledge this is the first HPLC procedure that can separate all of these compounds in a single chromatogram.

This study also shows that TEA can be used as a small ion-pair reagent in addition to its use as an amine modifier and that mobile phase pH and the concentration of ion-pairing reagents are two powerful parameters in the analysis of ionizable compounds by ion-pairing chromatography.

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