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HIGH-VOLTAGE ELECTROPHORETIC SEPARATION OF TRYPTOPHAN METABOLITES OF THE KYNURENINE PATHWAY

EEVA-LIISA SAINIO

Department of Pharmacology and Toxicology, University of Kuopio, P.O.B. 6, SF-70211 Kuopio (Finland)

and

PAAVO H. HYNNINEN*

Department of Chemistry, University of Helsinki, Vuorik. 20, SF-00100 Helsinki (Finland)

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SUMMARY

An analytical separation method for tryptophan and its seven metabolites of the kynurenine pathway by high-voltage paper electrophoresis is presented. Anthranilic acid, 3-hydroxyanthranilic acid, kynurenic acid, kynurenine, nicotinic acid, quinolinic acid, xanthurenic acid and unmetabolized tryptophan are measured in urine. Using radioactive labelling and scintillation counting as a quantification method, the relative standard deviation varied from 3.5% to 14.4%, corresponding to kynurenine and nicotinic acid, respectively. The recovery of labelled tryptophan added to urine was 95%. An advantage of the electrophoretic method is the minor tailing of spots and, hence, a good resolution of the components. For the monovalent anions of the kynurenine pathway metabolites, a linear correlation ($r=0.9996$) was found between the experimental relative electrophoretic mobility and the quantity $M^{-2/3}$, where M is the molecular mass of the anion.

INTRODUCTION

The metabolites of the kynurenine pathway of tryptophan (KPT) (Fig. 1) are of considerable biological and clinical significance [1–5]. Tryptophan 2,3-dioxygenase (E.C. 1.13.11) is thought to be the rate-limiting enzyme in the KPT and thus to regulate the formation of the metabolites. One of the most potent activators of tryptophan 2,3-dioxygenase is tryptophan itself [6]. Because many of the metabolites, such as xanthurenic acid, need vitamin B_6 for formation, the decreased concentration of xanthurenic acid in urine is considered to be a useful index of vitamin B_6 deficiency. Also of interest is the possible increased formation of 3-hydroxyanthranilic acid, which has been reported to be carcinogenic.

Analytical methods for the metabolites of the KPT are based on colour reactions [7,8], thin-layer chromatography (TLC) [9–11], gas chromatography (GC) [12,13] and recently on high-performance liquid chromatography (HPLC)

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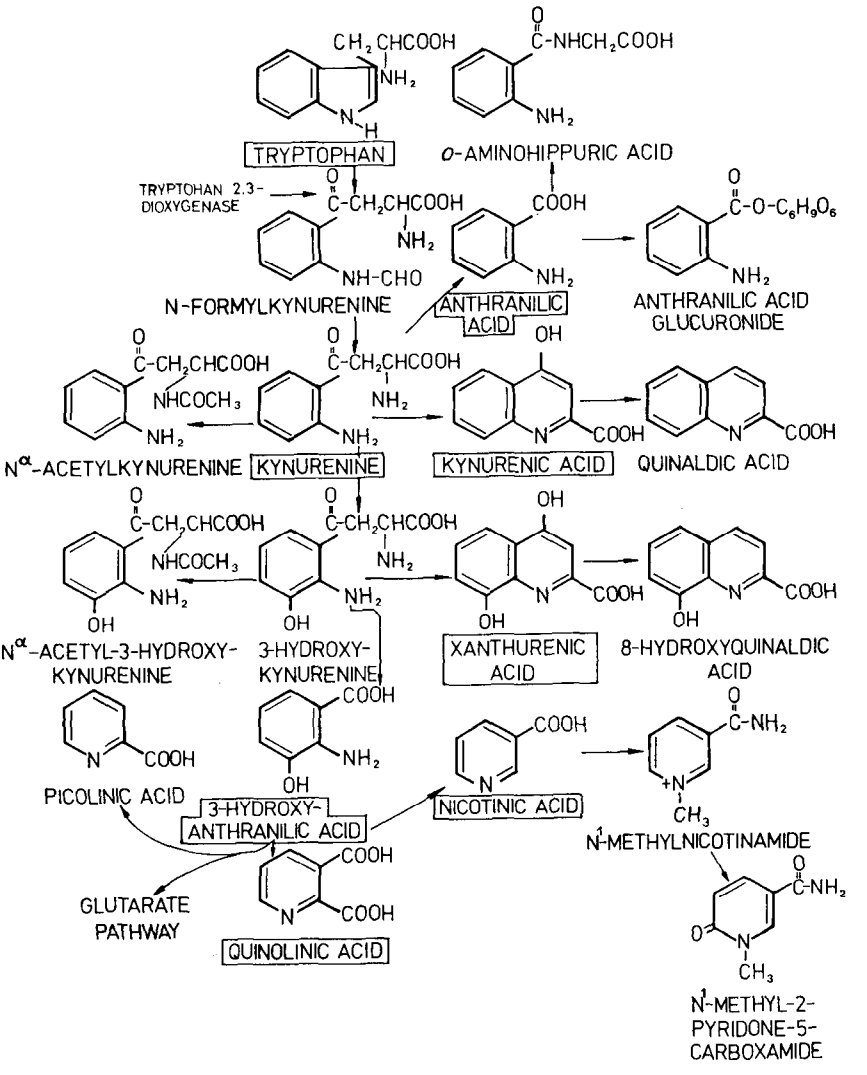

Fig. 1. The kynurenine pathway of tryptophan.

with spectrophotometric [14–17], fluorimetric [18] or electrochemical detection [19,20].

High-voltage electrophoresis (HVE) is suitable for the separation of low-molecular-mass ionic compounds, because the separation occurs with minimal diffusion. The method described in this paper is based on two-dimensional HVE on paper with the two developments in opposite directions. A similar HVE procedure has been reported for the separation of indole derivatives of the serotonin pathway [21].

THEORETICAL

Electrophoretic ion mobilities

The theory of electrophoresis has been reviewed by Edward [22] and by Morris and Morris [23]. In the following, only some basic equations, which are adequate for our purpose, will be considered.

Two fundamental equations characterize the ion mobility, u:

$$u=\frac{v}{E/L}=\frac{sL}{tE} \tag{1}$$

and

$$u=\frac{q}{6\pi\eta r} \tag{2}$$

where v is the velocity of the ion of charge q and radius r; s is the distance (cm) migrated by the ion during the development time t (s); E is the voltage (V) applied accross a support of length L (cm); and η is the viscosity of the liquid phase.

Eqn. 2 indicates that the ion mobility is directly proportional to the ion charge, q, but inversely proportional to the ion radius, r, and the viscosity, η, of the medium. Thus, cations and anions should move in opposite directions, and a divalent ion should migrate twice as far as a univalent ion, provided that the other parameters are kept constant. To achieve a resolution between two ions of similar charge, the difference of their radii should be as large as possible.

The principal difficulty in the use of eqn. 2 is the estimation of the ion radius, r. Because this equation assumes spherical particles, r could be calculated from the formula,

$$r=\left(\frac{3M\bar{v}}{4\pi N}\right)^{1/3} \tag{3}$$

where N is Avogadro's number, M is the molecular mass and $\bar{v}$ is the partial specific volume. However, partial specific volumes are seldom available in the literature for organic ions. In addition, it should be noted that $\bar{v}$ is expected to vary for different ionic forms of a compound, because various charged functional groups of the compound are hydrated differently. This makes the use of partial specific volumes for the calculation of ion mobility impractical. Similar difficulties are also encountered with the use of the diffusion constant, D, according to the following equation,

$$u=\frac{qD}{kT} \tag{4}$$

where k is the Boltzmann constant and T is the absolute temperature. Longsworth [24] has derived a simple semi-empirical relationship between the diffusion constant, D, and the molecular mass, M,

$$D=A/(M^{1/3}-B) \tag{5}$$

where A and B are constants. Using $A = 11.66 \cdot 10^{-6}$ and $B = 1.893$, Longsworth found that the calculated D values were within 5% of the experimental D values for 22 compounds, comprising amino acids, peptides and sugars.

On the basis of eqns. 4 and 5, a very simple relationship between the ion mobility and the molecular mass can be anticipated:

$$u = kzM^{-1/3} \tag{6}$$

where k is a constant and z is the electric charge in electron units. Eqn. 6 was first proposed by Offord [25], but it has received very little experimental support thus far. When studying the electrophoretic mobilities of a number of peptides on paper, Offord himself observed a slightly different equation to be valid for these compounds under the experimental conditions used:

$$u = k'zM^{-2/3} \tag{7}$$

A third relationship was proposed by Jokl [26]:

$$u = kzM^{-1/2} \tag{8}$$

Eqn. 8 is predictable on the basis of an old rule stating that the diffusion constant is inversely proportional to the square root of the molecular mass [27]. Using triethylammonium as a reference ion, eqn. 8 was quite well verified for 60 uni- and polyvalent organic anions and cations, with molecular masses in the range 32–500 [26]. A plot of the experimental "equivalent electrophoretic mobility", against $10^2 \cdot M^{-1/2}$, yielded a straight line for the studied ions [26].

Edward and Waldron-Edward [28] have explored the dependence of the free ion mobility, u_o, of several amino acids, amines, nitrophenols, etc., in infinitely dilute aqueous solution at 25 °C, upon the Van der Waals radius, r_w, and the frictional ratio, f/f_o, a shape factor. They derived the equation

$$u_o = \frac{1.14 \cdot 10^{-3} z}{r_w} \frac{f}{f_o} \tag{9}$$

where r_w was obtained from the Van der Waals volume ($V_w = 4\pi r_w^3/3$) calculated by the addition of the Van der Waals increments for the separate atoms or groups of the ion. Hydration was assumed to increase notably the Van der Waals increment of the $-COO^-$ group but not of the $-NH_3^+$ group [28]. In spite of the deviation of the axial ratio of the ion from 1, the shape factor was found to be very close to 1. Good consistency was found between experimental and theoretical relative paper electrophoretic mobilities [28].

In paper electrophoresis, the ion mobilities are reduced by an obstructive factor, $\xi < 1$, due to the tortuous paper network, and additionally by an adsorptive factor, $\rho \leqslant 1$, due to reversible adsorption of the ions on the cellulose fibres [28]:

$$u_p = \xi \rho u \tag{10}$$

These factors are very difficult to estimate quantitatively, because they depend on the chemical structure of the ion. However, they will be at least partly accounted for by the use of relative ion mobilities defined as follows:

$$u_r = u_p / u_{p,ref} \quad (11)$$

where $u_{p,ref}$ is the paper electrophoretic mobility of the selected reference ion. The introduction of the u_r also eliminates the effect of ion strength on the electrophoretic mobilities, although it should be noted again that this effect is not necessarily the same for all ions.

Ionization behaviour of the metabolites of the KPT

Because the electrophoretic mobility is roughly directly proportional to the electric charge of the ion, it is important to know the acid–base properties of the compounds to be analysed. The metabolites of the KPT are structurally quite diverse with several kinds of functional groups. Some of the derivatives [tryptophan (Trp) and kynurenine (Ky)] are amino acids and exhibit typical zwitterionic behaviour as a function of pH. Others are aromatic compounds with amino, carboxyl and hydroxyl substituents (Fig. 1). The presense of a "pyridine-type" nitrogen atom in several derivatives [kynurenic acid (KA), xanthurenic acid (XA), nicotinic acid (NA) and quinolinic acid (QA)] should also be noticed, because it participates in protonation–deprotonation equilibria at usual pH values. The approximate pK_a value for the KPT derivatives concerned in this paper, as well as the predicted principal ionic form of these derivatives at three different pH values, are given in Table I.

The examination of the pK_a values shows that all derivatives should exist as cations at pH 2 and would hence move towards the cathode. Their separation at this pH in one run would be difficult because all derivatives except Ky are present as monocations. Nevertheless, at this pH, Ky should be easily separable from Trp. At pH 7, the amino acids, Trp and Ky, would be virtually immobile, because their pI ($=6$) is close to this pH, while the other KPT derivatives would form anions via the deprotonation of their carboxyl groups. At this pH, the "pyridine-type" nitrogen would exist in deprotonated form. Thus QA should be easily separable from NA owing to its double negative charge compared with the latter. In principle, pH 12 could be also used for the separation of the KPT derivatives, but the separation of the three dianions [KA, 3-hydroxyanthranilic acid (HAA) and QA] would be expected to be difficult. From the various options, we have chosen a two-dimensional approach where the amino acids were run at pH 2 and the other derivatives were separated at pH 7.

EXPERIMENTAL

Chemicals and standard solutions

Standard solutions (0.5 mg ml^{-1}) were prepared from anthranilic acid (AA), HAA, KA, kynurenine sulphate, QA and XA (Sigma, St. Louis, MO, U.S.A.). A solution (2.5 mg ml^{-1}) was prepared of L-tryptophan (Fluka, Buchs, Switzerland). NA (Merck, Darmstadt, F.R.G.) was used at a concentration of 5.0 mg ml^{-1}. All chemicals except kynurenine sulphate and XA, were dissolved in acetone–water (1:1). To dissolve the latter chemicals completely, the pH of the solu-

TABLE I

ACID-BASE PROPERTIES OF TRYPTOPHAN AND ITS KYNURENINE PATHWAY METABOLITES

Compound	pK_a values [29-33]		Expected principal ionic form		
			pH 2	pH 7	pH 12
Anthranilic acid	pK_{HA} (ArCOOH)	3-5 (4.2, benzoic acid)	+	−	−
	pK_{BH}+ ($ArNH_2$)	3-5 (4.6, aniline)			
3-Hydroxyanthranilic acid	pK_{HA} (ArCOOH)	3-5	+	−	− −
	pK_{BH}+ ($ArNH_2$)	3-5			
	pK_{HA} (ArOH)	8-11 (10.0, phenol)			
Kynurenic acid	pK_{HA} (ArCOOH)	3-5	+	−	− −
	pK_{BH}+ (quinoline N)	5.4			
	pK_{HA}(ArOH)	8-11			
Kynurenine	pK_{HA} (α-COOH)	2.43	+ +	+ −	−
	pK_{BH}+ ($ArNH_2$)	3-5			
	pK_{BH}+ (α-NH_2)	9.44			
Nicotinic acid	pK_{HA} (ArCOOH)	4.84	+	−	−
	pK_{BH} + (pyridine N)	5.04			
Quinolinic acid	pK_{HA} (ArCOOH)	3-5	+	− −	− −
	pK_{BH}+ (pyridine N)	5.04			
Tryptophan	pK_{HA} (α-COOH)	2.43	+	+ −	−
	pK_{BH}+ (α-NH_2)	9.44			
	pK_{BH}+ (indole NH)	−2.38			
Xanthurenic acid	pK_{HA} (ArCOOH)	3-5	+	−	− − −
	pK_{BH}+ (quinoline N)	5.4			
	pK_{HA} (ArOH)	8-11			

tions was adjusted to 8 with 2 *M* sodium hydroxide. L-[5(n)-^{3}H]tryptophan (TRK 460) was purchased from Amersham (U.K.).

Solvents for electrophoresis

Solvent A. A potassium phosphate (0.02 *M*)-potassium chloride (0.14 *M*) solution was used in electrophoretic procedure 1. The pH was adjusted to 7.00 with potassium hydroxide.

Solvent B. An aqueous solution (pH 2.00) of formic acid (3.5 vol.%) and acetic acid (10 vol.%) was used in electrophoretic procedure 2.

Electrophoretic apparatus and procedures

The separations were performed with a Shandon L24 high-voltage electrophoresis apparatus for analytical and preparative applications.

Procedure 1. The standards (5 μl) and urine (10 μl) were applied on a 61 cm × 23 cm piece of Whatman No. 1 paper, at 15 cm from the cathode edge. The cathode and anode edges of the plate were connected with solvent A in the electrophoresis chamber by means of paper strips wetted with the solvent. Cold water was run in the jacket around the electrophoresis chamber to prevent the temperature of the medium from rising too high and to avoid condensation. The voltage was 3000 V and the current was 20 mA. Development time was 3 h. Excluding Ky

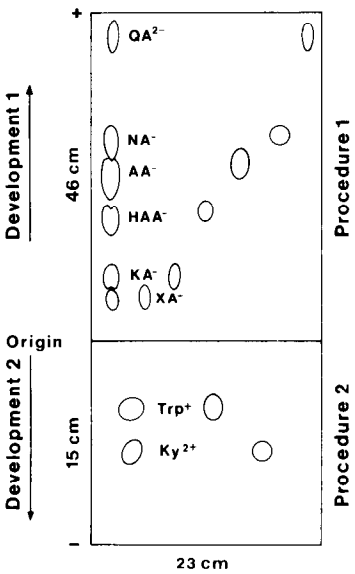

Fig. 2. The high-voltage electrophoregram of tryptophan and its kynurenine pathway metabolites. Spots: XA = xanthurenic acid; KA = kynurenic acid; HAA = 3-hydroxyanthranilic acid; AA = anthranilic acid; NA = nicotinic acid; QA = quinolinic acid; Trp = tryptophan; Ky = kynurenine.

and Trp, all other metabolites migrated towards the anode when solvent A was used. After development and drying overnight at room temperature, the fingerprints were localized on the paper under UV light ($\lambda = 254$ nm) and photographed. Then part A (41 cm × 23 cm) was cut off and, for the quantification of the metabolites, the spots on it were separately cut off (see Fig. 2). After slicing into small strips, each spot was placed into a scintillation counting bottle and the counting was performed with a Wallac scintillation counter.

Procedure 2. Here, the part that was cut off in procedure 1 was replaced with an equal-size piece of clean Whatman No. 1 paper. Solvent B was used in this procedure. The voltage applied was again 3000 V and the current was 35 mA. The electropherogram was developed in the opposite direction to that in procedure 1 (see Fig. 2). The development time was 1.5 h. In other respects, this procedure was as procedure 1.

Quantitative determination of the metabolites in urine

To evaluate the utility of HVE for clinical and biological purposes, quantitative determinations of the KPT metabolites from rat urine were performed. A male Wistar rat, weight 200 g, was given twice a dose of 20 μCi of ^{3}H-labelled Trp with 6 h between the injections, concurrently with a load of 200 mg kg^{-1} of unlabelled Trp, also given twice. For the collection of urine, the rat was kept for 9 h in a metabolic cage made of transparent polycarbonate plastics. The urine was collected in a vessel containing 1 ml of 0.5% oxalic acid. The sample was centrifuged and stored at -20°C until use.

The recovery of Trp from urine was measured by adding 50 nCi of labelled Trp

TABLE II

ELECTROPHORETIC ION MOBILITIES OF TRYPTOPHAN AND ITS KYNURENINE PATHWAY METABOLITES

Ion	pH	M	10^3 M^{-1}	10^2 $M^{-\frac{1}{2}}$	10^2 $M^{-\frac{1}{3}}$	10 $M^{-\frac{1}{3}}$	$10^4\ u^{exptl}$★ ($cm^2\ s^{-1}\ V^{-1}$)	u_r^{exptl}	$10^4\ u^{theor}$★★ ($cm^2\ s^{-1}\ V^{-1}$)	u_r^{theor}
XA^-	7.0	204.16	4.898	2.884	6.982	1.698	0.0834	0.2117	3.393	0.8572
KA^-	7.0	188.16	5.315	3.045	7.290	1.745	0.1266	0.3214	3.454	0.8727
HAA^-	7.0	152.13	6.573	3.509	8.108	1.873	0.2442	0.6200	3.665	0.9260
AA^-	7.0	136.13	7.346	3.779	8.571	1.944	0.3256	0.8265	3.738	0.9444
NA^-	7.0	122.10	8.190	4.063	9.050	2.016	0.3939	1.0000	3.958	1.0000
QA^{2-}	7.0	165.10	6.057	3.323	7.783	1.823	0.6029	1.5306	7.379	1.8643
							(0.3014)★★★	(0.7653)	(3.690)	(0.9322)
TrP^+	2.0	203.22					0.0491	1.0000	3.276	1.0000
Ky^{2+}	2.0	206.20					0.0768	1.5660	6.590	2.0116
							(0.0384)★★★	(0.7830)	(3.295)	(1.0058)

★Calculated from Eqn. 1; figures represent the arithmetic mean of two determinations.
★★Calculated from Eqn. 9; $f/f_o = 1$; hydration of the $-COO^-$ group has not been taken into account.
★★★Equivalent mobility $= u/z$.

to normal urine and comparing the counts with those yielded by an equal dose of Trp directly applied to the paper.

RESULTS AND DISCUSSION

The electrophoretic patterns obtained for the metabolites of the KPT by two-dimensional HVE are shown in Fig. 2. A complete separation was achieved by procedure 1 for AA, HAA, KA, NA, QA and XA. The amino acids, Ky and Trp, were easily resolved by procedure 2. NA and Trp were selected as reference ions for the anions and cations, respectively. The experimental mobilities were calculated from eqn. 1 and the theoretical ones from Edward's formula, eqn. 9. Two different series of r_w values were used in the latter calculation. In one series hydration of the $-COO^-$ group was taken into account, but in the other series this effect was not considered. The value of the frictional ratio was assumed to be 1 in eqn. 9.

Comparison of the experimental relative ion mobilities (u_r^{exptl}) with the theoretical ones (u_r^{theor}) in Table II shows that the former have considerably lower values than the latter in the instance of XA^- and KA^{-1}. Consideration of the hydration did not diminish the discrepancy between theory and experiment and even increased it. The observed inconsistency between theory and experiment may be interpreted in terms of the obstructive factor (ξ) and the adsorptive factor (ρ) (see eqn. 11) which may attain notably different values for the large molecules compared with the relatively small reference ion, NA^-.

To study the applicability of eqns. 6–8, the values of M^n were calculated for $n = -1$, $-2/3$, $-1/2$. or $-1/3$ (Table II). When these were plotted against the relative experimental mobilities, it was found that the best linear fit was obtained

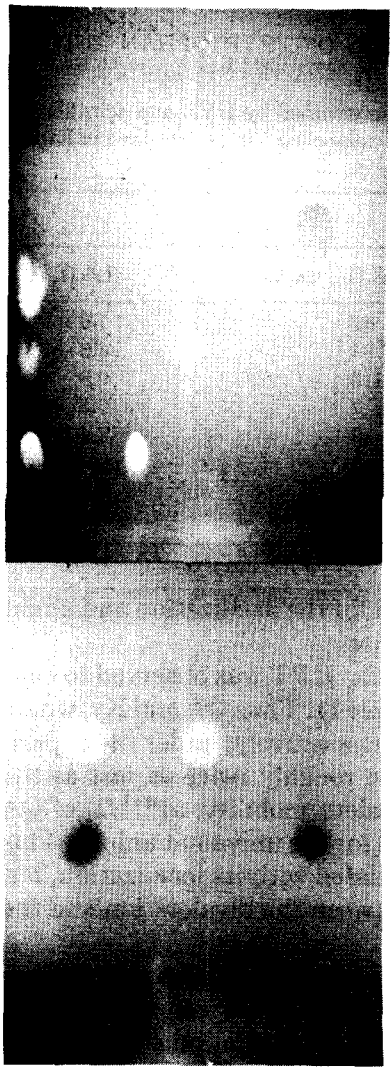

Fig. 3. Photograph of the high-voltage electrophoregram corresponding to Fig. 2. The upper and lower parts of the figure do not match precisely, because they were photographed separately with different magnifications.

with $n = -2/3$ (equation of the regression line, $y = 0.6727x - 1.7288$; the product moment correlation coefficient, $r = 0.9996$; $y = u_r^{exptl}/z$, $x = 10^2 M^{-2/3}$). However, because the other n values also resulted in almost comparable fits, it may be concluded that the determination of relative ion mobilities was not precise enough. We measured the mobilities from the midpoints of the spots and calculated the mean of two determinations from separate runs. It should also be noted that the precision of the experimental ion mobilities depends on the localization technique of the separated components. Here, the fingerprints on the electrophoretic paper were made visible under UV light and photographed (Fig. 3). Autoradio-

TABLE III

PRECISION OF THE QUANTITATIVE DETERMINATION OF TRYPTOPHAN AND ITS KYNURENINE PATHWAY METABOLITES

Tryptophan and its kynurenine pathway metabolites were determined by HVE and scintillation counting from the urine of a rat that had received labelled and unlabelled Trp as described in Experimental. n = number of determinations of each metabolite.

Compound	10^{-3} DPM		
	Mean	S.D. ($n=5$)	R.S.D. (%)
Anthranilic acid	595	42	7.0
3-Hydroxyanthranilic acid	1403	84	6.0
Kynurenic acid	1142	113	9.9
Kynurenine	201	7	3.5
Nicotinic acid	83	12	14.4
Quinolinic acid	n.d.	n.d.	n.d.
Tryptophan	1107	58	5.2
Xanthurenic acid	635	56	8.8

graphy or fluorescence detection could also be used for the localization and might improve the precision of the experimental mobilities.

The ionization behaviour of the metabolites of the KPT was observed to conform to the pK_a values of the ionizable groups (Table I). Thus QA and Ky, which are expected to exist as a dianion and a dication, respectively, under the experimental conditions used, were observed to migrate roughly twice as fast as the monovalent ions (Table II). The observed equivalent mobility, u_r^{expt1}/z, of QA does not fit the linear correlation found to be valid for the univalent anions. This deviation may be caused by the uncertainty associated with its localization, but may be due to the absence of adsorptive forces between the divalent ion and the support.

To study the applicability of two-dimensional HVE to quantitative determination of the KPT metabolites from urine, five separate urine samples collected from the same rat (cf. Experimental) were analysed. Scintillation counting was used for quantitation of the radioactively labelled metabolites from the separated spots. The relative standard deviation (R.S.D.) varied from 3.5% (Ky) to 14.4% (NA) (cf. Table III). When tritium labelling is used, it should be noticed that the localization of labelling in Trp affects the results [34]. In the radioactive Trp used in this work, the benzene ring was labelled. This part of the Trp molecule is probably metabolized last (Fig. 1). In addition, the sensitivity of this quantitation method depends on the dose of radioactive Trp used. For the recovery of Trp from urine, a value of 95.2% (the mean of six determinations) was obtained.

Two-dimensional HVE offers a valuable alternative to the chromatographic methods previously used for the same analytical purposes. An improved resolution of the components seems evident compared with that achievable by paper and thin-layer chromatography, where the Trp metabolites occur in neutral form. The neutral forms of these compounds are liable to form relatively strong hydrogen bonds owing to the presence of carboxyl and hydroxyl groups in the mole-

cules. Also π–π interactions are possible owing to the delocalized π-electrons of the aromatic rings. These interactions can be expected to cause aggregation of the solute molecules or their strong adsorption on supports such as paper and cellulose acetate. Both phenomena lead to excessive broadening and tailing of the solutes, thus impairing the resolution. Aggregation and adsorption are less likely to occur in HVE owing to the repulsion between similarly charged ions and solvation of ions. Among the various assay methods used for Trp metabolites, HPLC is considered to be "the method of choice, as it affords rapid and efficient assays of these compounds at their endogenous levels with minimal sample pre-treatment" [20]. This opinion appears to be well justified, particularly if one considers the excellent resolution obtained recently by Krstulović et al. [20] using an improved isocratic HPLC technique. In spite of this, we believe that HVE is a good alternative and may be even superior to HPLC with respect to unusual Trp metabolites, e.g. QA. QA is a Trp metabolite that is determined only seldom. In the reversed-phase HPLC system used by Tarr [17], it was eluted as a broad band hardly distinguishable from the baseline. A likely explanation for this situation is found in the high aggregation tendency of QA in its neutral form, owing to the presence ot two –COOH groups. Its separation and determination present no difficulties using HVE. We consider that the HVE method described here should also be applicable to routine analyses in clinical laboratories.

REFERENCES

1 H.A. Krebs, in K.F. Gey and L.A. Carlson (Editors), Metabolic Effects of Nicotinic Acid and Its Derivatives, Hans Huber, Bern, 1971, p. 1115.
2 H. Wolf, J. Clin. Lab. Invest., 33 Suppl. 136 (1974) 63.
3 F.A.G. Teulings, H.A. Peters, W.C.J. Hop, W. Fokkens, W.G. Haije, H. Portengen and B. van der Werf-Messing, Int. J. Cancer, 21 (1978) 140.
4 G. Sulochana and R. Arunagiri, Ind. J. Exp., Biol., 16 (1978) 507.
5 M. Watanabe, T. Takahashi, M. Yoshida, M. Suzuki and S. Muramatsu, J. Nutr. Sci. Vitaminol., 25 (1979) 115.
6 W.E. Knox and A.H. Mehler, J. Biol. Chem., 187 (1950) 419.
7 S.L. Tompsett, Clin. Chim. Acta, 4 (1950) 411.
8 N.C. Chen and R.K. Gholson, Anal. Biochem., 47 (1972) 139.
9 T. Diamantstein and H. Ehrhart, Hoppe-Seyler's Z. Physiol. Chem., 326 (1961) 131.
10 C.A. Benassi, F.M. Veronese and E. Gini, J. Chromatogr., 14 (1964) 517.
11 M.P. Walsh, Clin. Chim. Acta, 11 (1965) 263.
12 H. Wegman, H.-Ch. Curtius and U. Redweik, J. Chromatogr., 158 (1978) 305.
13 J.M. van der Poll, M. Vink, J. Schrijver and J. Odink, J. Chromatogr., 375 (1986) 213.
14 E. Grushka, E.J. Kikta, Jr. and E.W. Naylor, J. Chromatogr., 143 (1977) 51.
15 M. Ghebregzabher, S. Rufini, M.G. Castellucci and M. Lato, J. Chromatogr., 222 (1981) 191.
16 I. Morita, T. Masujima, H. Yoshida and H. Imai, Anal. Biochem., 118 (1981) 142.
17 J.B. Tarr, Biochem. Med., 26 (1981) 330.
18 F. Geeraerts, L. Schimpfessel and R. Crokaert, Clin. Chim. Acta, 102 (1980) 247.
19 A.M. Krstulović, M.J. Friedman, P.R. Sinclair and J. Felice, Clin. Chem., 27 (1981) 1291.
20 A.M. Krstulović, M.J. Friedman, H. Colin, G. Guiochon, M. Gaspar and K.A. Pajer, J. Chromatogr., 297 (1984) 271.
21 B.N. Johri, J. Chromatogr., 50 (1970) 340.
22 J.T. Edward, Adv. Chromatogr., 2 (1966) 63.

23 C.J.O.R. Morris and P. Morris, Separation Methods in Biochemistry, Pitman, London, 2nd edn., 1976, p. 705.
24 L.G. Longsworth, J. Am. Chem. Soc., 74 (1952) 4155.
25 R.E. Offord, Nature (London), 211 (1966) 591.
26 V. Jokl, J. Chromatogr., 13 (1964) 451.
27 I.M. Hais, Chem. Listy, 49 (1955) 709.
28 J.T. Edward and D. Waldron-Edward, J. Chromatogr., 20 (1965) 563.
29 J.D. Rawn, Biochemistry, Harper & Row, New York, 1983, p. 50.
30 J. March, Advanced Organic Chemistry. Reactions, Mechanisms, and Structure, 2nd ed., McGraw-Hill, New York, 1977, p. 225.
31 P. Sykes, A Guidebook to Mechanism in Organic Chemistry, Longman, London and New York, 5th ed., 1981, p. 52.
32 W.O. Foye (Editor), Principles of Medicinal Chemistry, Lea & Febiger, Philadelphia, PA, 2nd ed., 1981, p. 901.
33 R. Stewart, The Proton: Applications to Organic Chemistry, Academic Press, Orlando, FL, 1985, pp. 9–145.
34 B.K. Madras and T.L. Sourkes, Arch. Biochem. Biophys., 125 (1968) 829.