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SURFACTANT ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF TRYPTOPHAN AND SOME OF ITS METABOLITES IN BIOLOGICAL FLUIDS

C.M. RILEY, E. TOMLINSON*, T.M. JEFFERIES and P.H. REDFERN

School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath (Great Britain)

SUMMARY

A rapid, sensitive assay for tryptophan and some of its metabolites in urine, plasma and saliva has been developed using sodium dodecylsulphate as a pairing ion in a surfactant ion-pair high-performance liquid chromatography technique. The method is highly selective for tryptophan which is separated from its main indoleamine metabolites, 5-hydroxytryptophan, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid, and from kynurenine. The usefulness of the assay has been demonstrated in plasma level and urinary excretion studies of orally administered tryptophan.

INTRODUCTION

Tryptophan is metabolised by two primary routes, either through the tryptophan—kynurenine—nicotinic acid pathway [1] or through a series of indoleamines [2]. Abnormal plasma and urine levels of metabolites formed by either pathway have been implicated in diseased states [3—9].

Tryptophan blood levels have been considered [10] as being indicative of mood changes in man and there are several reports of the benefits of tryptophan in the treatment of depression [11, 12]. Tryptophan is found in significant amounts in saliva and it has been suggested [13] that there is an equilibrium between free tryptophan in plasma and in saliva. We are currently investigating whether salivary levels of tryptophan can be used as an indicator of depressive states.

A variety of methods have been employed for the determination of tryptophan in biological fluids, including thin-layer chromatography [14, 15], amino-acid analysis [16—18], gas—liquid chromatography [19, 20], UV spectrometry

*To whom correspondence should be addressed. Address until August 1979: College of Pharmacy, The Ohio State University, Columbus, Ohio 43210, U.S.A.

[21], and fluorimetry [22—23]. These methods have proved to be insensitive, time consuming and/or fairly non-selective. Recently, reversed-phase high-performance liquid chromatography (HPLC) [24, 25] and ion-pair HPLC [26] methods have been presented for the analysis of tryptophan and proved to be highly selective. Due to the ability of tryptophan to ionise in acidic solutions, a reversed-phase surfactant ion-pair HPLC system has been used in this study. Such techniques have been shown to have advantages over conventional reversed-phase techniques in terms of flexibility of retention control, peak shape, speed of analysis and to be appropriate for the assay of solutes in biological fluids [27—31].

EXPERIMENTAL

Apparatus

This consisted of a Haskell constant-pressure pneumatic amplifier liquid pump (Olin Energy 24030). Dectection was by UV spectroscopy by either a Jobling fixed-wavelength (280 nm) UV detector (LD 1205) or a Cecil variable-wavelength UV detector (CE 212). The detector output was recorded on a potentiometric detector (Bryans 28000). Injections were made on column with a Specac 8 port-injection valve fitted with a 5 μl internal loop and a 100 μl internal external loop. The column and injection valve temperature were controlled by enclosure in a modified gas-chromatograph oven (Perkin Elmer F11).

Materials

The stationary support was ODS-silica (5 μm Spherisorb ODS) packed into a 100 × 5 mm (I.D.) stainless-steel tube by a dilute slurry technique [32]. The pure samples of tryptophan and its metabolites were from Sigma (Poole, Great Britain). The mobile phase consisted of 50% methanol (HPLC grade, Fisons, Loughborough, Great Britain) in 0.04 mole dm^{-3} di-sodium hydrogen citrate buffer containing decyl, dodecyl or tetradecyl sodium sulphate (Cambrian Chemicals, Croydon, Great Britain). The pH of the mobile phase was adjusted to 2.25 by the addition of 2 *N* hydrochloric acid using a pH meter. Potassium nitrate was used as an unretained compound for the determination of capacity ratios.

Procedures

Samples of urine, blood and saliva were taken from healthy male volunteers. The urine was injected untreated, but the saliva and blood were pretreated to remove cellular matter and protein. The cellular components of blood were removed by centrifugation at 600 *g* for 10 min and the plasma drawn off with a Pasteur pipette. The high molecular weight components of saliva and plasma were removed by centrifugation filtration through a CF 50A Centiflo membrane at 2000 *g* for 20 min, as described by Bauman et al. [33]. This method removed all molecules of molecular weight above 50,000.

Estimations of free levels of tryptophan only were made. It was found that storage of biological samples containing tryptophan (even if frozen at −11°) was not possible, due to the rapid degradation of tryptophan. This is consistent with the findings of Grushka et al. [25], thus all biological samples were chromatographed as soon as possible after collection and preparation.

RESULTS AND DISCUSSION

This work was designed to develop an assay for tryptophan in biological fluids and forms part of a series of investigations into the potential and mechanisms of surfactant ion-pair HPLC [34, 35]. It has previously been demonstrated [34, 35] that chromatographic conditions in surfactant ion-pair HPLC can be optimised by investigating the effect of changing the mobile phase concentration and alkyl chain length of the pairing ion.

Fig. 1 shows the sigmoidal relationship between the alkylsulphate mobile phase concentration and the capacity ratio of tryptophan. This relationship has been attributed [31] to the mixed retention mechanism in surfactant ion-pair HPLC, being a combination of ion-pair distribution and in situ ion exchange [28, 36]. Fig. 2 shows the linear relationship between the logarithm of the

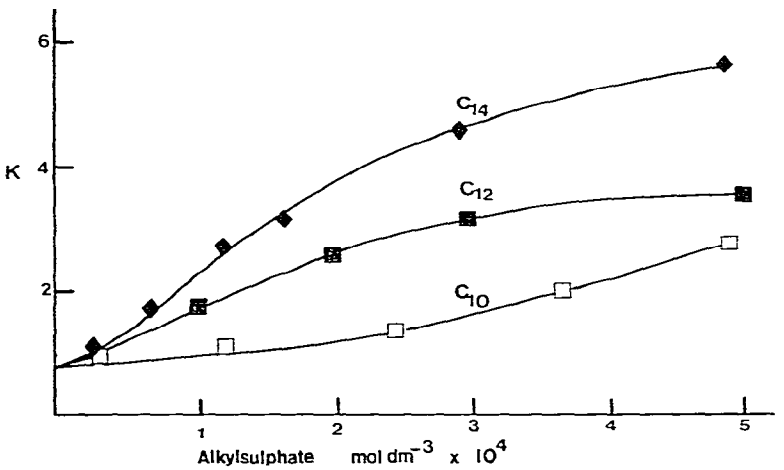

Fig. 1. The relationship between the capacity ratio (κ) of tryptophan and the concentration of decyl (C_{10}), dodecyl (C_{12}) and tetradecyl (C_{14}) sodium sulphate pairing ions in the mobile phase. Conditions: temperature, 30°; flow-rate, 2.0 ml min^{-1}; stationary support, 100 × 5 mm (I.D.) ODS-silica (5 μm); mobile phase, 50% methanol in citrate buffer at pH 2.25 containing alkylsulphate pairing ions.

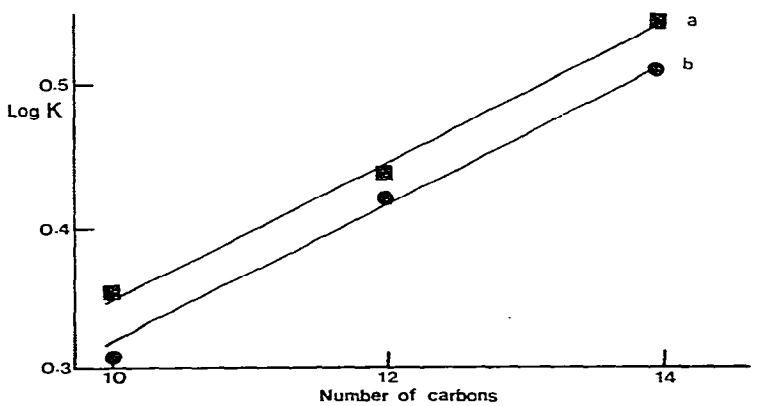

Fig. 2. The relationship between the logarithm of the capacity ratio of tryptophan and the number of carbons in the alkylsulphate pairing ions at mobile phase concentration of: (a) $5.0 \cdot 10^{-4}$ mole dm^{-3}; (b) $4.5 \cdot 10^{-4}$ mole dm^{-3}. Conditions as Fig. 1.

capacity ratio of tryptophan and the number of carbons in the alkylsulphate pairing ion. From Figs. 1 and 2 it is demonstrated that the required capacity ratio for a solute molecule can be achieved by choosing the appropriate pairing ion concentration and chain length, without any further alteration to the mobile phase composition. The assay development was at 30° and the actual determinations in biological fluids were at ambient temperature. The reduction in temperature resulted in a rise in capacity ratio for trypthophan from 3.52 to 4.00 for mobile phase concentration of $4 \cdot 10^{-4}$ mole dm^{-3} dodecyl sodium sulphate.

Fig. 3 shows how column efficiency in surfactant ion-pair HPLC systems is related to the homologue concentration, with high values of reduced plate height (h) being obtained at low pairing ion concentration. Maximum column efficiency is obtained above a $3 \cdot 10^{-4}$ mole dm^{-3} pairing ion concentration. These results are produced below the critical micelle concentration of the pairing ion and it is likely that at higher concentrations micellisation of the pairing ion in the mobile phase will alter column efficiency and capacity ratios.

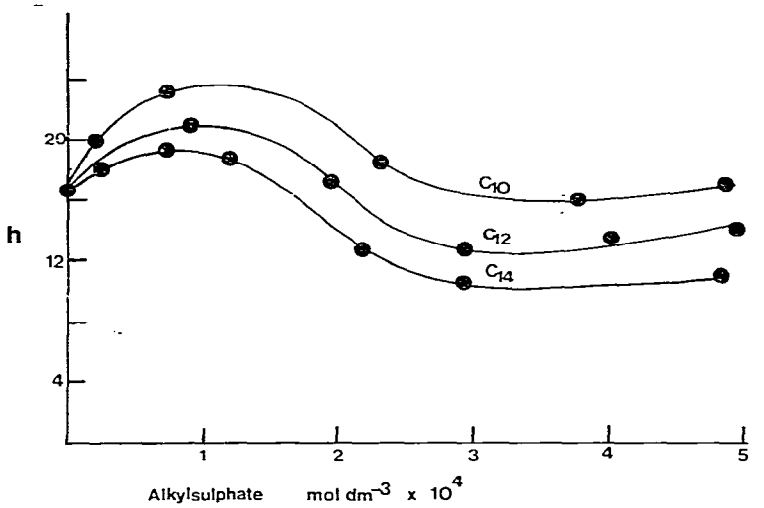

Fig. 3. The effect of alkylsulphate pairing ion concentration and chain length on the reduced plate height (h). Conditions as Fig. 1.

For the analysis of tryptophan in biological fluids, a mobile phase concentration of $4 \cdot 10^{-4}$ mole dm^{-3} sodium dodecylsulphate was used since this resulted in an ideal capacity ratio value for tryptophan and acceptable column efficiency.

All estimations of tryptophan were made on the basis of peak height determinations and by comparison with known standard solutions. The linearity of this response was checked by calibration curves in aqueous solutions and in spiked samples of plasma and saliva (see Table I). The calibration curve for tryptophan in plasma did not pass through the origin due to the presence of endogenous tryptophan in the plasma. The amount of tryptophan in the plasma controls was calculated by extrapolation of the line and was found to be $2.2 \cdot 10^{-3}$ mole dm^{-3} (see Fig. 4). Tryptophan-free saliva controls were obtained by rinsing the mouth with a citric acid solution.

TABLE I

SUMMARY OF THE CALIBRATION PLOTS OF TRYPTOPHAN AGAINST PEAK HEIGHT OR PEAK HEIGHT RATIO IN VARIOUS ENVIRONMENTS

Tryptophan concn. range (mole dm^{-3})	Internal standard concn. (mole dm^{-3})	Environment	Number of points	Correlation coefficient (r)
10^{-5}—10^{-4}	4-Aminophenol (10^{-4})	Aqueous buffer	5	0.996
10^{-6}—10^{-5}	—	Aqueous buffer	5	0.996
10^{-5}—10^{-4}	4-Chloroanaline (3×10^{-4})	Plasma	4	0.997
10^{-6}—10^{-5}	—	Saliva	5	0.987

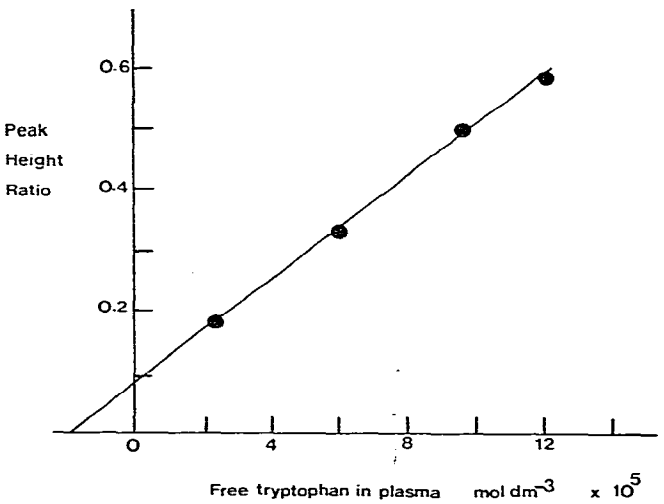

Fig. 4. The relationship between the peak height ratio of tryptophan to internal standard, and the concentration of tryptophan in plasma. Conditions: Temperature, ambient; flow-rate, 2.0 ml min^{-1}; mobile phase, 50% methanol in citrate buffer at pH 2.25 with $4 \cdot 10^{-4}$ mole dm^{-3} sodium dodecylsulphate.

The coefficient of variation of the peak heights produced by ten consecutive 10-μl injections of a 10^{-5} mole dm^{-3} tryptophan solution was 1.68% indicating that four point assays were possible without internal standards, provided that a loop valve injector was used. For aqueous solutions, 4-aminophenol (which eluted before trypthophan) was used as internal standard. For biological fluids, 4-chloroanaline (which eluted after tryptophan) was used as internal standard. For the assays the same internal standard was used in the reference solution as in the biological fluids (see Table I for concentrations of internal standards used). The limit of detection was 10^{-10} moles and for determinations of tryptophan below 10^{-5} mole dm^{-3} injections of 100 μl were required. This resulted in band broadening and incomplete resolution of tryptophan and 4-chloroanaline, so that the internal standard was omitted from these determinations.

The selectivity of the method was demonstrated by injecting controls of plasma, urine and saliva (Fig. 5) and by separation of tryptophan from its metabolites 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT), 5-hydroxy-3-indoleacetic acid (5-HIAA) and kynurenine (Fig. 6). The tryptophan-free saliva contained no peak corresponding to tryptophan. The peaks corresponding to tryptophan in urine and plasma were identified by the addition of authenticated samples of tryptophan.

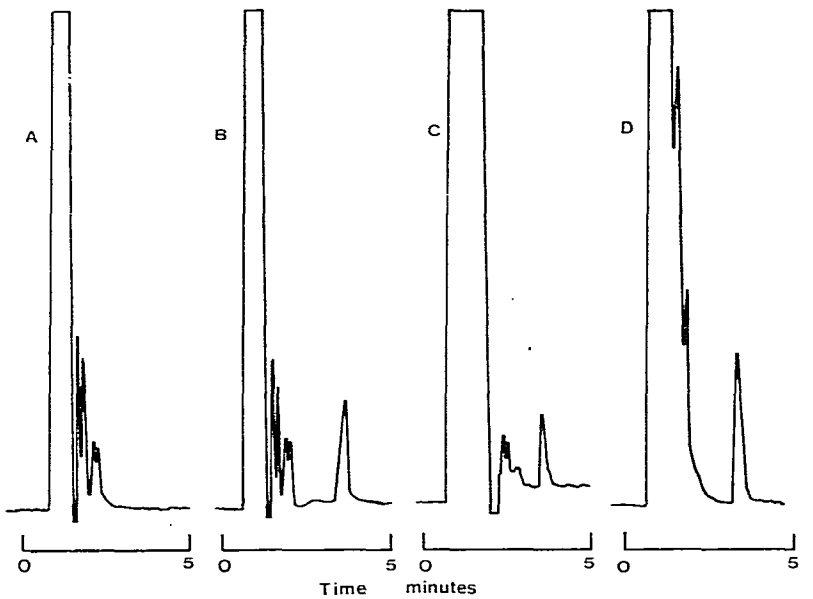

Fig. 5. Chromatographic traces of biological fluids. A = Tryptophan free saliva; B = saliva spiked with $4 \cdot 10^{-6}$ mole dm^{-3} tryptophan; C = plasma; D = urine. Conditions as Fig. 4.

In general, metabolites of biological substances are more polar than the parent compound since this facilitates urinary excretion by the kidney. Fig. 7 shows the important metabolitic pathways of tryptophan. The gain of a hydrophilic substitutent —OH causes a dramatic fall in capacity ratio from 4.0 to 1.5. The loss of an uncharged —COOH to give 5-HT has a lesser effect reducing the capacity ratio from 1.5 for 5-HTP to 1.2 for 5-HT. The principle of retention in surfactant ion-pair HPLC involves both hydrophobic interactions and the interaction between a charged group in the solute molecule (in this case $\equiv\overset{+}{N}-$) and an oppositely charged group ($-SO_4^-$) in the pairing ion. Loss of the charged amino group to give 5-HIAA has the greatest effect on retention such that 5-HIAA is almost unretained having a capacity ratio of 0.5. Splitting of the hydrophobic indole ring to give kynurenine is partly compensated for by the fact that kynurenine has two free charged amino groups available for interaction and so has a capacity ratio of 2.2. This group contribution approach is useful not only in rationalising results but also in the prediction of retention of compounds, particularly if authentic samples are unavailable, as is often the case when drug metabolites or degradation products are under investigation.

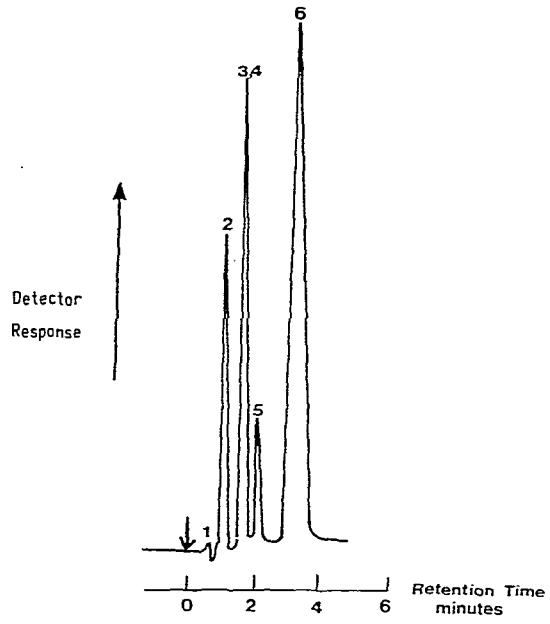

Fig. 6. Chromatographic trace to show the separation of tryptophan from its main metabolites: 1 = solvent (κ = 0); 2 = 5-HIAA (0.5); 3 = 5-HT (1.2); 4 = 5-HTP (1.5); 5 = kynurenine (2.2); 6 = tryptophan (4.0). Conditions as Fig. 4.

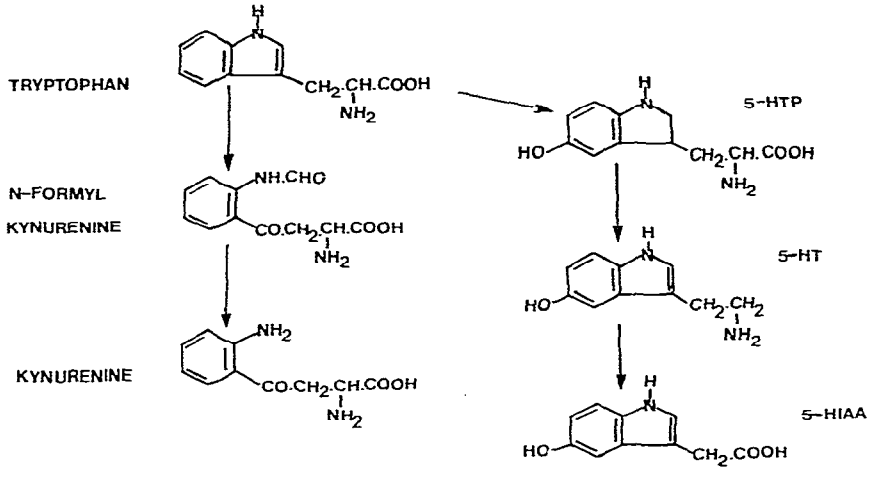

Fig. 7. Summary of the main metabolic pathways of tryptophan.

The urinary excretion profile and bioavailability of tryptophan after a single oral loading dose were determined in fasting male subjects using the developed assay. For the urinary excretion study 1 g (i.e. 2 × 500-mg Optimax tablets) was administered orally and urine samples taken at intervals over a period of 6 h. For the bioavailability study, 2 g (i.e. 4 × 500-mg Optimax tablets) was administered orally and 10 ml blood samples and 10 ml saliva samples taken at

intervals over a period of 6 h. Saliva was collected over a period of 10 min with no attempt made to stimulate salivation artificially. Table II shows the concentration of tryptophan found in the urine and the total amounts of tryptophan excreted with time. Table III shows the concentration of free tryptophan found in plasma and saliva with time. These results confirm those obtained by Ashley et al. [37] in a similar study using fluorimetry as an assay technique, although higher levels of sensitivity have been achieved in this present work.

Salivary levels of tryptophan correlate well with the levels found in plasma, which reinforces the hypothesis that the tryptophan in saliva is in equilibrium with the free tryptophan in plasma. The pharmacological significance of these findings will be reported elsewhere.

TABLE II

THE CONCENTRATION OF TRYPTOPHAN AND THE TOTAL AMOUNT OF TRYPTOPHAN EXCRETED IN THE URINE WITH TIME AFTER A 1 gm ORAL DOSE

Time (h)	Tryptophan concentration in urine		Volume of urine taken	Cumulative amounts of tryptophan excreted	
	(mole $cm^{-3} \cdot 10^4$)	($\mu g\ ml^{-1}$)	(ml)	(moles $\cdot 10^5$)	(mg)
0	1.38	27.9	90	1.24	2.51
0.62	1.22	24.7	80	2.22	4.49
1.55	3.64	73.6	54	4.18	8.45
2.87	1.74	35.2	195	7.58	15.33
5.42	2.09	42.3	71	9.67	19.55

TABLE III

CONCENTRATION OF FREE TRYPTOPHAN IN PLASMA AND SALIVA WITH TIME AFTER A 2 gm ORAL DOSE

Time (h)	Plasma concentration		Time (h)	Saliva concentration	
	(mole $dm^{-3} \cdot 10^5$)	($\mu g\ ml^{-1}$)		(mole $dm^{-3} \cdot 10^5$)	($\mu g\ ml^{-1}$)
0	0.78	1.57	0	0.14	0.28
0.58	1.49	3.02	0.92	0.19	0.38
1.08	2.23	4.51	1.72	0.28	0.57
1.70	2.82	5.71	3.42	0.22	0.44
4.58	1.06	2.14	4.78	0.13	0.27
5.58	0.59	1.19			

CONCLUSIONS:

A surfactant ion-pair HPLC assay which uses low concentrations of pairing ion has been shown to be an effective one for the assay of tryptophan in biological fluids, and should permit the possible salivary/blood level correlations for tryptophan to be well investigated. The flexibility of the approach also permits the various metabolites of similar structure to be well resolved. The study has also provided us with group contribution data for ion-pair systems for possible use in predicting solute retention behaviour.

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