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Note

# Thin-layer chromatographic method for the quantitative analysis of L-tryptophan in human plasma

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Considerable interest in monitoring plasma L-tryptophan levels during treatment with anti-inflammatory drugs has been awakened by Aylward and co-workers [1—3].

During an investigation into the possible interaction between aspirin and diclofenac (an anti-inflammatory drug marketed by Ciba-Geigy, Basle, Switzerland), the plasma levels of salicylic acid, diclofenac and L-tryptophan were monitored. In addition, several other parameters in the plasma had to be monitored to satisfy the protocol requirements of this clinical trial. These requirements made it necessary to develop simple analytical methods by which salicylic acid and L-tryptophan could be quantitatively measured with precision and accuracy, employing the smallest possible volume of plasma in order to avoid drawing excessive amounts of blood.

Although numerous methods exist for the determination of L-tryptophan [4—14], none of them were found to be entirely suitable, mainly from the point of view of sample requirement or because the method was deemed too cumbersome and time-consuming. Previous experience with thin-layer chromatographic (TLC) methods involving measurement of in situ fluorescence of the spots on the TLC plate [15, 16] prompted an investigation into the possibility of using such a method for the determination of L-tryptophan. This paper describes a TLC method by means of which L-tryptophan can be determined in plasma. Only 50 $\mu$l of plasma are required if the same aliquot is used to determine salicylic acid in the sample as well.

## EXPERIMENTAL

### *Reagents*

All reagents used were of guaranteed reagent grade (Merck, Darmstadt, G.F.R.) and were used without further purification. L-Tryptophan was obtained from Merck, and also from Sigma (St. Louis, Mo., U.S.A.), and was used as received.

### *Apparatus*

A Perkin-Elmer MPF3 spectrofluorimeter equipped with a TLC scanning attachment was used to measure the fluorescence of the spots on the TLC plates using the following conditions: light source, xenon lamp; excitation wavelength, 287 nm; emission wavelength, 335 nm; excitation slit width, amplifier sensitivity and sample adjustment were set so as to obtain approximately 80% full-scale deflection on the recorder when the strongest spot in the chromatogram was being scanned.

The other apparatus used consisted of a Shandon S/P Chromatank, Hamilton dosing syringes (10 $\mu$l and 50 $\mu$l), 5-$\mu$l disposable glass capillary micropipettes calibrated at 1 $\mu$l intervals (Clay Adams Division of Becton Dickenson & Co., Parsipany, N.Y., U.S.A.), and a Desaga Autospotter.

### *Stock solutions*

A single stock solution containing 1.00 mg of L-tryptopan per millilitre of solution was prepared by making 50 mg of L-tryptophan up to 50 ml in 0.1 *N* NaOH. This solution was kept in a refrigerator at 4°.

### *Plasma standards*

Fresh human plasma (10 ml) was shaken with 100 mg of activated charcoal for 5 min and then filtered under pressure through a Celite bed (1 cm high) in a disposable plastic syringe. This procedure was shown to remove tryptophan quantitatively from the plasma. Four standard solutions of L-tryptophan were prepared by adding, with a Hamilton syringe, 5, 10, 15 and 20 $\mu$l of the stock solution in each instance to 1 ml of the charcoal-treated plasma contained in plastic-capped sample tubes. Corrections were applied to compensate for the small volume changes. These standard solutions were kept frozen at —20° when not in use.

### *Preparation of plasma samples for chromatography*

A 50-$\mu$l volume of plasma (standard or unknown) was measured accurately with the dosing syringe into a small plastic-stoppered, disposable, conical centrifuge tube. To the plasma were added 100 $\mu$l of methanol in order to precipitate the proteins partially. After thorough mixing on a vortex mixer, the stoppered tube was centrifuged for 1 min to produce a clear supernatant.

### *Spotting the plates*

A 3-$\mu$l aliquot of the clear supernatant was spotted on cellulose TLC plates without fluorescence indicator (Merck) in three applications of approximately 1 $\mu$l at a time, while drying with a hairdrier between applications. In this fash-

ion sixteen spots with unknown (X) and standard (S) alternating in duplicate were applied to a single 10 cm × 20 cm plate ($S_1X_1S_2X_2S_3X_3S_4X_4$). Alternatively, 6 $\mu$l were spotted using the Desaga Autospotter.

*Chromatography*

The mobile phase used was *n*-butanol—acetone—glacial acetic acid—water (35:35:7:23). Ascending development was carried out in an unsaturated tank up to a height of 6.5 cm; the elution time was ca. 20 min. The plates were dried briefly with a hairdrier and then allowed to stand open to a clean atmosphere at room temperature for 15 min before being scanned. (Drying in an oven at 100° was found to have a deleterious effect on the baseline of the densitograms.) The $R_F$ value of L-tryptophan was found to be 0.52.

Each spot was then scanned in the TLC scanning attachment of the $MPF_3$ spectrofluorimeter in the direction of the solvent flow. Standard curves were constructed by plotting peak height versus plasma concentration of the known standards. An equation for the best straight line fit was obtained by linear regression analysis and this equation used to calculate the plasma concentrations of unknowns by substitution of the peak heights of the unknowns into the equation.

## RESULTS AND DISCUSSION

Fig. 1 represents part of a densitogram showing the peaks obtained for standard plasma containing the indicated amount of L-tryptophan, plasma samples obtained from two different persons, as well as a blank plasma after charcoal treatment. The second peak at $R_F$ 0.29 (indicated by an asterisk in the densitogram) was obtained in all normal plasma samples investigated. Generally, a good linear relationship existed between the concentration of L-tryptophan (5—20 $\mu$g/ml) and peak height so that an equation of the best straight line fit could be found by linear regression analysis ($r^2 > 0.98$ normally)

A summary of the recoveries of L-tryptophan added to charcoal-treated plasma is presented in Table I.

To ascertain whether it would be feasible to use aqueous standards of L-tryptophan instead of standards made up in plasma, standards of L-tryptophan were prepared in water in exactly the same way as described for the plasma. These were then treated in the same way as the plasma standards and spotted on the same plates alternating with the plasma standards. The results obtained are illustrated in Fig. 2 in which were plotted the peak heights of plasma standards versus peak heights corresponding to aqueous standards for adjacent spots on the chromatogram. A good linear correlation between the peak heights for the two types of standards was apparent but the slope indicated that L-tryptophan would be underestimated by a factor of 1.06 if aqueous standards were used. This factor was shown to be constant over a period of one year, suggesting that aqueous standards could be used provided a correction factor were applied.

The effect of scanning the spots across the direction of the solvent flow was investigated. The results are given in Table I. An example of a chromato-

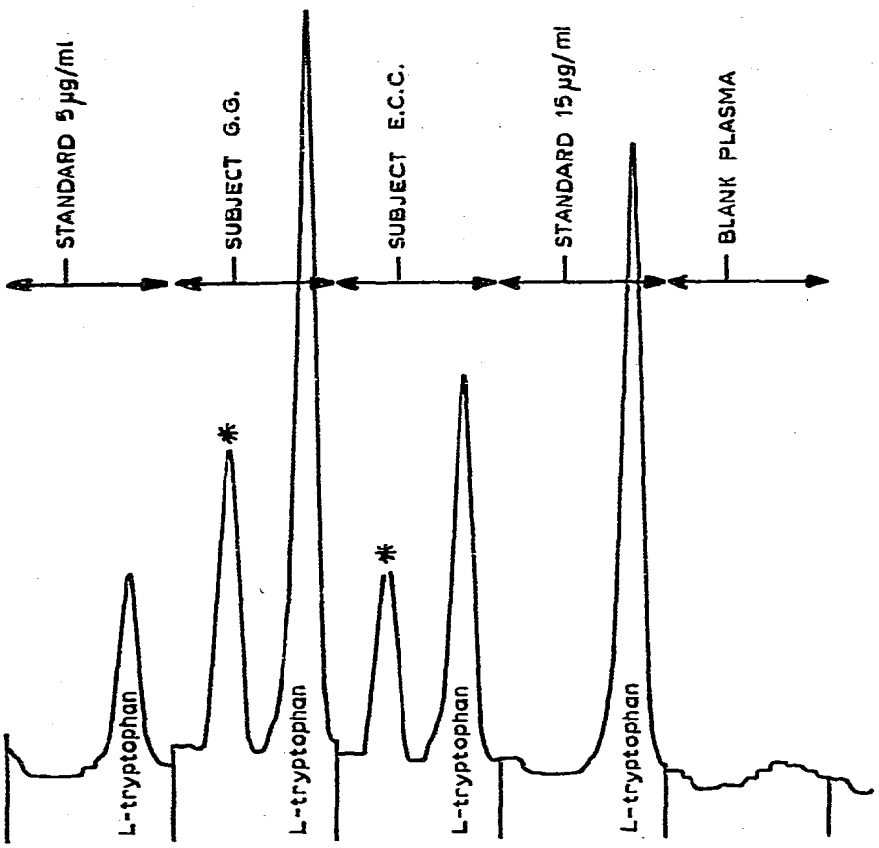


**Fig. 1. Densitogram of L-tryptophan in plasma of two volunteers and in plasma standards. Asterisk (*) indicates peak ($R_F$ 0.29) due to endogenous substance present in all normal plasma samples.**

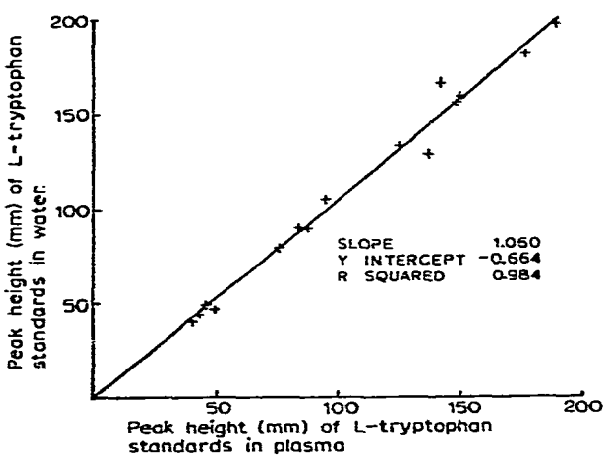


**Fig. 2. Comparison of L-tryptophan standards in plasma with L-tryptophan standards in water.**

TABLE I

RECOVERY OF L-TRYPTOPHAN FROM HUMAN PLASMA

| Method* | L-Tryptophan added (μg) | Mean recovery (μg) | C.V. (%) | N |
|---|---|---|---|---|
| a | 8.00 | 8.18 | 5.50 | 4 |
| | 11.00 | 10.74 | 3.45 | 4 |
| | 14.00 | 13.97 | 7.59 | 4 |
| | 17.00 | 17.32 | 6.18 | 4 |
| b | 8.00 | 7.85 | 7.39 | 4 |
| | 11.00 | 10.45 | 10.43 | 4 |
| | 14.00 | 13.53 | 3.62 | 4 |
| | 17.00 | 17.59 | 2.96 | 4 |
| c | 5.00 | 4.39 | 9.11 | 4 |
| | 12.50 | 12.45 | 8.59 | 4 |
| | 17.50 | 16.52 | 8.60 | 4 |
| | 20.00 | 19.02 | 2.72 | 4 |

*(a) Spots scanned in direction of solvent flow; (b) spots scanned across direction of solvent flow; (c) spots applied with Desaga Autospotter.

gram scanned across the direction of solvent flow is presented in Fig. 3. From the results obtained it can be seen that the chromatograms can be scanned across the direction of solvent flow, with considerable saving of time, without significant difference in accuracy or precision.

For comparison with the manual spotting method, spiked plasma samples were treated as before but 6 μl of the supernatant were spotted using the Desaga Autospotter. A summary of the recoveries of L-tryptophan in this case is presented in Table I. It is evident that lower accuracy and precision were attained using the Autospotter.

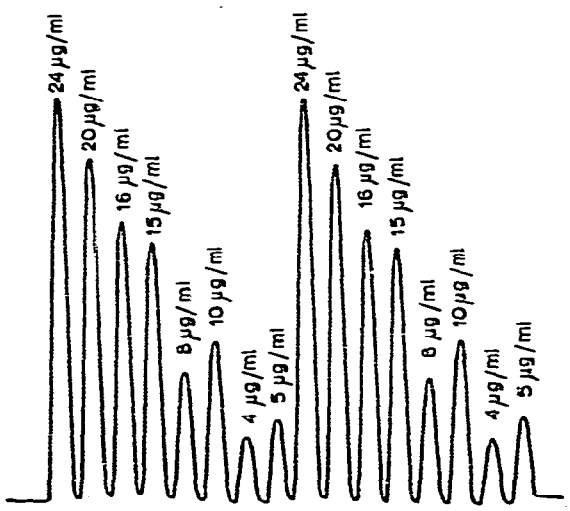


Fig. 3. Densitogram of spiked plasma samples scanned across the direction of solvent flow.

The optimum excitation and emission wavelenghts were found to be quite critical. In contrast with assays of many other compounds performed by in situ fluorescence measurement on thin-layer plates, cut-off filters for the emitted light could not be used in this case.

No interference with the determination was observed with the following compounds tested: mephenamic acid, flufenamic acid, niflumic acid, thiaprofenic acid, nalidixic acid, flubiprofen, fenoprofen, ketoprofen, ibuprofen, indoprofen, fenbufen, alclofenac, diclofenac, oxyphenbutazone, phenylbutazone, naproxen, probenecid, sulindac, indomethacin, penicillin G, furosemide, piretinide, all at a concentration of 20 μg/ml; paracetamol at 50 μg/ml; and salicylic acid at a concentration of 300 μg/ml. Likewise, no interference from metabolites of salicylic acid was experienced.

L-Tryptophan plasma levels (mean 12.33 μg/ml, range 7.15—19.94 μg/ml, S.D. 2.59) determined in 20 normal volunteers taking no medication are within the range reported by other workers [1, 10—14].

This method was used to determine L-tryptophan in a large number of plasma samples obtained from volunteers participating in a clinical trial involving the ingestion of aspirin. Determination of salicylic acid in the plasma samples was carried out by TLC on silica gel plates using the same supernatant aliquot described above. The small amount of sample required for the assay (the determination can be scaled down to 5 μl of plasma) makes the method ideally suitable for the study of L-tryptophan in plasma or other biological fluids where only small volumes of plasma are available.

Two recent publications [17, 18] on the determination of L-tryptophan in plasma came to our attention while this manuscript was being prepared.

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