CAPSULES
notes and applications from Bioanalytical Systems, Inc.

Determination of Tryptophan Metabolites by LCEC

Tryptophan (Trp) is metabolized via a number of important pathways and serves as a major amino acid building block for protein biosynthesis. Perhaps the most significant, as well as the most studied, role of tryptophan is as the precursor of serotonin (5-HT) (Figure 1). Serotonin is a potent neurotransmitter and vasoconstrictor of great neurochemical interest. It has been implicated in a variety of behavioral functions ranging from thermoregulation to depression. The accurate measurement of serotonin levels in body tissues and fluids is thus of great interest to neurochemists and behavioral scientists. From a clinical standpoint, plasma levels of 5-HT are of particular interest as these may serve as a diagnostic tool for verifying certain types of carcinomas (1).

The major catabolite of serotonin, 5-hydroxyindoleacetic acid (5-HIAA), is of considerable interest also, primarily as an indicator of 5-HT metabolism. 5-HIAA is clinically useful as urine from patients with malignant carcinoid, a tumor of the enterchromaffin tissue, contains elevated amounts of 5-HIAA.

The methodology for quantitating tryptophan metabolites depends a great deal on the sample matrix, which determines the degree of sample cleanup required prior to the final analysis. For this reason, methods will vary in their degree of complexity depending on the nature of the original sample.

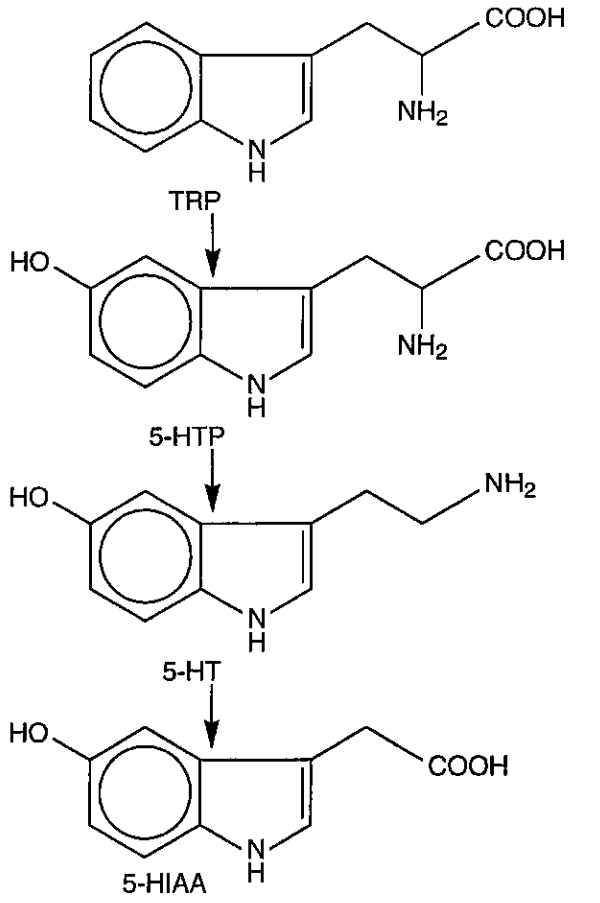

Figure 1. *Metabolism of tryptophan along the serotonin pathway.*

Brain Tissue and CSF

Methods for determining 5-HT and/or 5-HIAA in brain tissue and CSF have typically utilized fairly extensive sample clean up steps. Extraction of 5-HT into butanol followed by back extraction into 0.1 M HCl is a common approach (2). This does not allow for convenient determination of 5-HIAA in the same sample, however, and requires significant time for work up. The use of small gravity-fed ion exchange columns is a more versatile approach as the determination of 5-HT and 5-HIAA from the same sample is readily accomplished. (3-4) It is also possible to incorporate a step for isolation of tryptophan into this sequence. Unfortunately this approach also suffers from considerable time requirements.

More recently it has been recognized that the determination of 5-HT and 5-HIAA in brain tissue and CSF can be conveniently accomplished with no sample cleanup beyond deproteinization of homogenate supernatants. (5-7) The scheme which we have found convenient is outlined in Figure 2. This simple approach is made possible by the relatively clean sample matrix combined with the excellent selectivity of the LCEC system at the chosen operating potential.

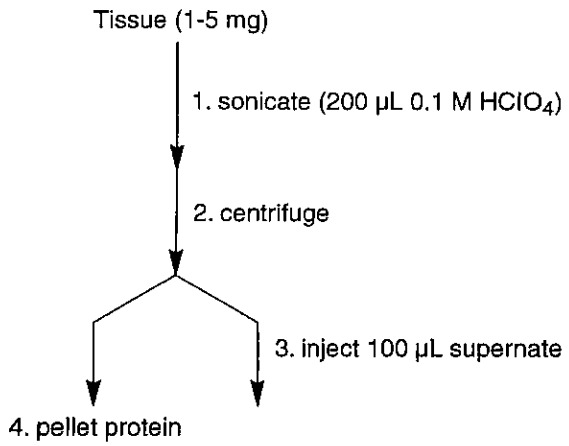

Figure 2. *Schematic of method for simple determination of 5-HT and 5-HIAA in brain tissue.*

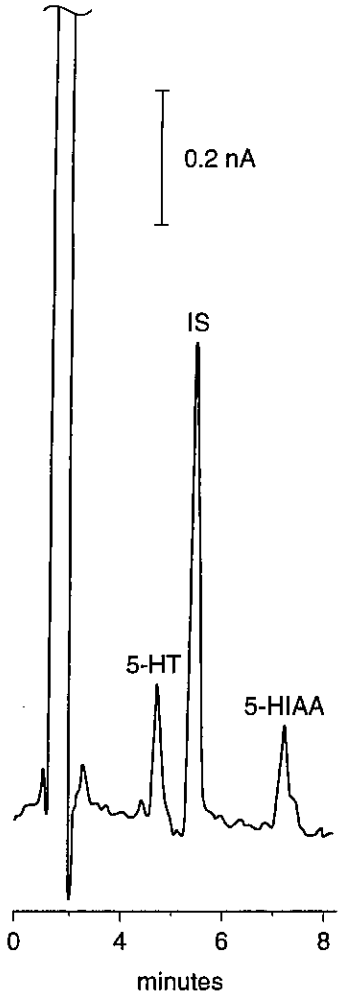

Figure 3. *Determination of 5-HT and 5-HIAA in punchout from rat hypothalamus by procedure outlined in Figure 2. Punchout was made from a 450 µm slice using an 18 gauge needle.*

By simplifying sample preparation according to the outlined scheme the following benefits are gained: (1) analysis time is reduced, (2) sample loss is minimized, and (3) handling of small tissue or fluid samples is facilitated. With respect to the third point, we have utilized this procedure for the simultaneous determination of 5-HT and 5-HIAA in punchouts from brain tissue (Figure 3). Sample sizes ranged from 1-5 mg. The ability to handle very small samples is particularly important in that it allows quantitation in very specific regions of the brain.

Plasma and Urine

As mentioned earlier, the complexity of the matrix determines the degree of sample cleanup necessary. In plasma (serum) and urine the matrix is such that significant cleanup is required. It may be possible to quantitate 5-HT and 5-HIAA without extensive sample handling but the analysis time will be considerably longer and the potential for extraneous interferences much greater.

We have found the use of ion-exchange resins to be the most widely applicable and convenient for isolating 5-HT and 5-HIAA from these matrices. Again tryptophan may be incorporated conveniently into the scheme. Figure 4 outlines the isolation sequence developed by Koch and Kissinger (3). The methodology is well suited to both plasma and urine samples and is very effective in isolating the desired compounds. (Figure 5).

The determination of 5-HT is plasma is further complicated by binding of 5-HT to platelets. Thus two pools of plasma 5-HT exist, bound and unbound. If it is desired to quantitate bound as well as unbound 5-HT, an additional step must be inserted into the scheme of Figure 4 to achieve platelet disruption and free the bound 5-HT. This is most conveniently accomplished by sonication (8). Another important point is that collection of plasma is critical and will effect the results dramatically. If plasma is collected by simple centrifugation then both the speed and time are factors which must be accounted for as the platelet concentration in the resultant plasma will be influenced by these parameters.

Trace Enrichment

In some cases the use of gravity fed isolation columns can be a problem due to sample dilution in the isolation step. This may be a problem when analyte levels are extremely low or when sample size is limited. One approach to overcoming this difficulty is the

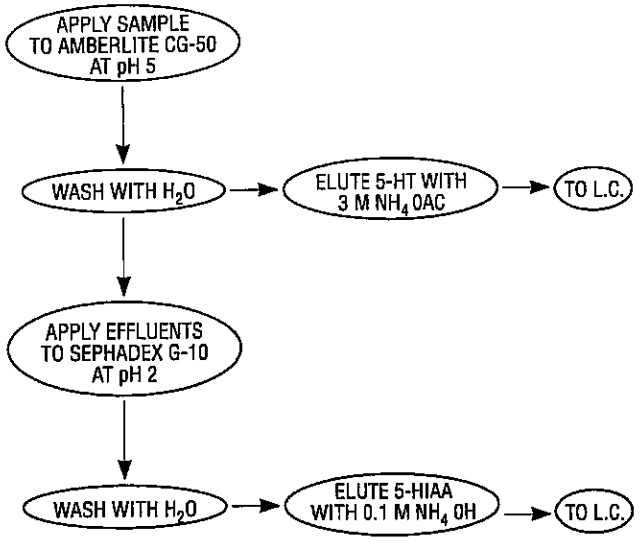

Figure 4. *Isolation scheme for quantitation of 5-HT and 5-HIAA in urine or plasma.*

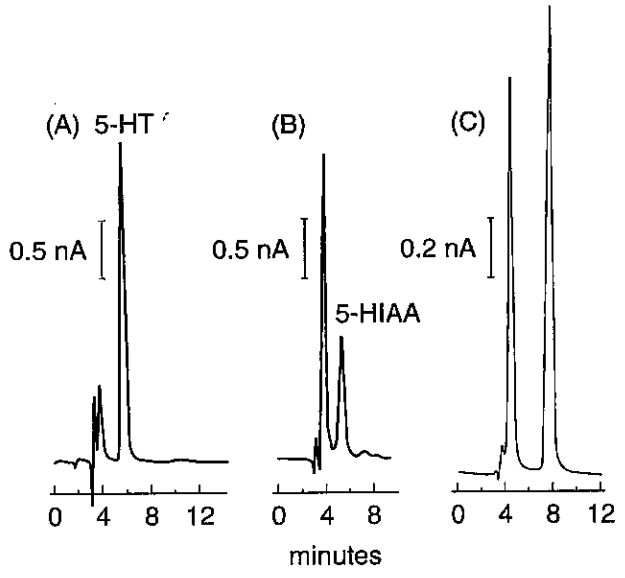

Figure 5. *Determination of (a) free 5-HT, (b) 5-HIAA, (c) Trp from 1 mL plasma using the scheme of Figure 4 for isolation. Conditions: (a) + (b) instrument: BAS LC-154; column: Waters μBondapak C_{18}, 4 x 300 mm; mobile phase: 85% 0.5 M NH_4OAC, pH 5.1, 15% MeOH; flow rate: 1.0 mL/min; detector: TL-3(CPO), +500 mV. (c): same as (a) + (b) but mobile phase was 80% 0.1 M citrate/phosphate (McIlvaine) buffer, pH 4.0, 20% MeOH. For Trp isolation the effluent from the Amberlite is applied to a Dowex AG-50 column and eluted with 0.1 M NH_4OH. The Dowex effluents are then applied to Sephandex G-10 as outlined.*

use of microcolumns packed with the appropriate resins in order to minimize elution volumes (4). The difficulty here is that extensive handling of small volumes is tedious, and very small volumetric losses are large in terms of percentages.

An alternative approach to the sample dilution problem is the use of trace enrichment for on-line preconcentration of the sample prior to final quantitation. A schematic of the basic apparatus is shown in Figure 6. In this configuration the LC is capable of handling injected volumes of 1-2 mL without degrading chromatographic performance. Koch et al. successfully utilized this approach for quantitation of tryptophan metabolites in small tissue and fluid samples (9).

In the trace enrichment method, analytes of interest are pumped onto the cartridge (C1) in mobile phase 1 (M1) and after an appropriate time interval are eluted onto the analytical column (C2) for final quantitation by mobile phase 2 (M2). The critical parameters are the selection of C1, M1, M2, and the switching interval. For the tryptophan metabolites selection of C1 and M1 is simple. Their native hydrophobicity is

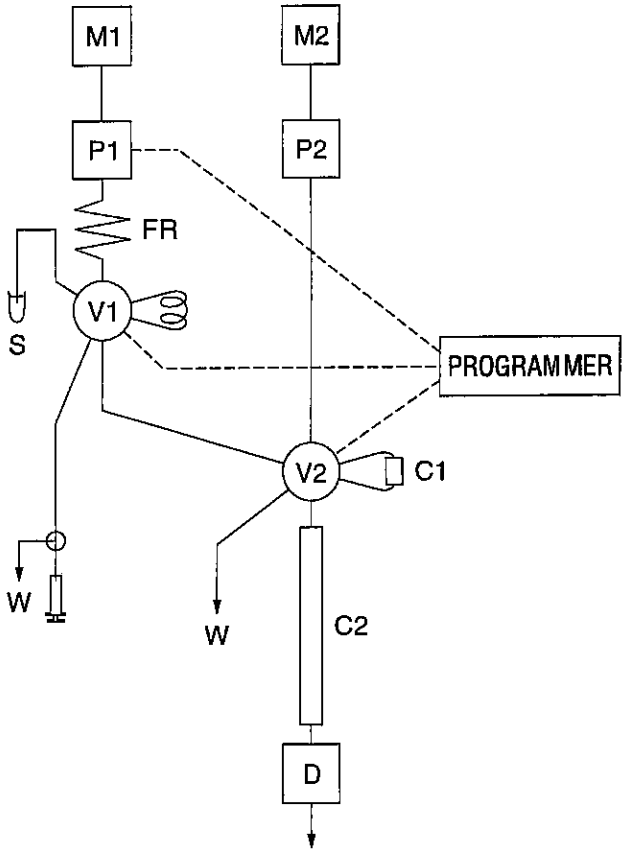

Figure 6. *Schematic of trace enrichment system.*

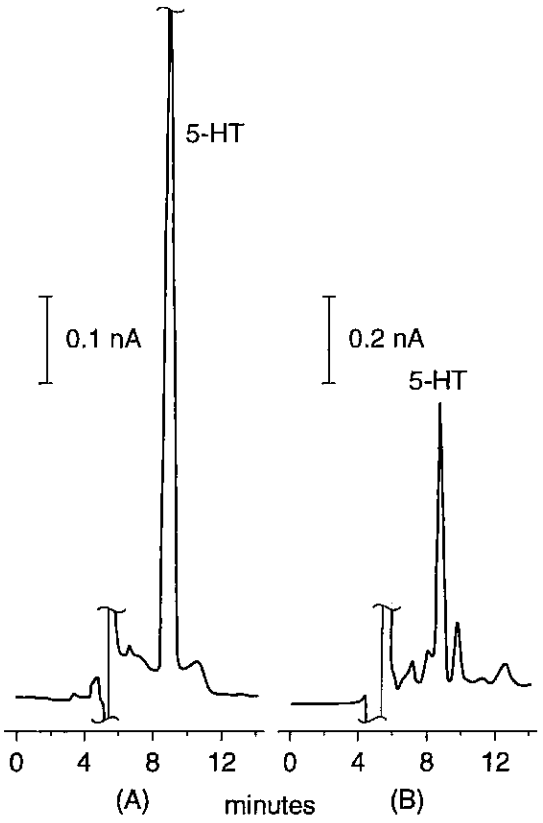

Figure 7. *Determination of free 5-HT from a) 1 mL and b) 100 µL of plasma using the isolation scheme of Figure 4 followed by the trace enrichment for quantitation. Conditions: C1: Merck RP-18, 4.6 x 30 mm; M1: 100% 0.42 M NH_4OAc, pH 5.1: Flowrate: 1.8 mL/min; inj volume: 2.0 mL; C2: waters µBondapack C_{18},4 x 300 mm; M2: 85% 0.5 M NH_4OAC, pH 5.1, 15% MeOH, Flowrate 1 mL/min; Detector: TL-3 (CPO, +500 mV).*

such that standard C18 packing material and a simple aqueous buffer work very well for preconcentration. The switching valve (V2) is then activated. The preconcentrated analytes are eluted with an aqueous buffer containing methanol and the final separation is made on the analytical column, also C18. Effective utilization of trace enrichment requires the use of highest purity mobile phase components in order to avoid preconcentration of interfering impurities.

Additional Comments

The preceding discussion dealt with the determination of 5-HT and 5-HIAA since these two metabolites are of primary interest and considerable work has been done in this area. Nonetheless, many other tryptophan metabolites, as well as tryptophan itself, are compatible with LCEC assays (7). Other metabolites may require higher operating potentials and thus decrease the selectivity of the LCEC approach. This results in a greater burden on the system and magnifies the need for sample cleanup. Direct injection of brain homogenate supernatants, for example, is not practical at a detector potential greater than 800-850 mV. If trypotophan or other metabolites requiring operation above this range are of interest then it may be necessary to utilize intermediate isolation steps. This will depend largely on the desired sensitivity.

Related BAS Publications

1. *Current Separations, Vol. 2, No. 2 (1980), Applications of LCEC to Neurochemical Analysis.*
2. *Capsule No. 203, Tissue Catecholamines.*
3. *Capsule No. 204, HVA and DOPAC in Brain Tissue.*
4. *Capsule No. 227, Plasma Catecholamines.*
5. *Capsule No. 228, Urinary Catecholamines by LCEC.*
6. *Capsule No. 231, Urinary Metanephrines.*
7. *Capsule No 229, Urinary Vanilylmandelic Acid.*
8. *Capsule No. 230, Urinary Homovanillic Acid.*
9. *Capsule No. 233, Enzyme Activity by LCEC. CMOT, DBH, PNMT, Tyr H, Trp H, AAD.*
10. *Bibliography of Recent Reports on Electrochemical Detection.*
11. *Capsule No. 186, Serotonin in Blood.*

References

1. *Bio-Science Handbook, Bio-Science Laboratories, 12th ed., Van Nuys, CA (1979) 98.*
2. *S. Sasa and C.L. Blank, Anal. Chem, 49 (1977) 354-359.*
3. *D.D. Koch and P.T. Kissinger, J. Chromatogr., Biomed. Applications, 164 (1979) 441-455.*
4. *W.H. Lyness, N.M. Friedle, and K.E. Moore, Life Sci., 26 (1980) 1109-1114.*
5. *I.N. Mefford and J.D. Barchas, J. Chromatogr., Biomed. Applications. 181 (1980) 187-193.*
6. *J.F. Reinhard, M.A. Moskowitz, A.F. Sved, and J.D. Fernstrom, Life Sci., 27 (1980) 905-912.*
7. *W.A. Jacobs, Current Separations (Bioanalytical Systems), 2(2) (1980).*
8. *S. Sasa, C.L. Blank, D.C. Wenke, and C.A. Sczupak, Clin. Chem., 24 (1978) 1509-1514.*
9. *D.D. Koch and P.T. Kissinger, Life Sci., 26 (1980) 1099-1107.*