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Note

# Comparison of high-performance liquid chromatography and gas chromatography—mass spectrometry for the analysis of indole-3-acetic acid in brain tissue

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Tryptamine, a presumably neuroactive metabolite of tryptophan, is one of the so-called trace amines [1] whose exact role in the mammalian central nervous system has not been elucidated to date. Existing experimental data suggest that it plays a role in the behavioural syndrome produced by administration of tryptophan with an inhibitor of monoamine oxidase [2] and that its effects resemble those of lysergic acid when infused in man [3]. Also, it has been shown that its actions in cerebral cortex [4] and hypothalamus [5] are opposite to those exerted by serotonin (5-hydroxytryptamine). Lately, a high-affinity binding site for [$^3$H]tryptamine has been described [6]. Thus, all experimental evidence supports a neuroregulatory role for tryptamine in the central nervous system. However, the concentration of this amine in rat brain is far below the levels of the classical neurotransmitter amines, such as serotonin and catecholamines [7]. Indole-3-acetic acid (IAA), the main metabolic product of tryptamine, could be taken as an index of the functional activity of tryptamine in the central nervous system [8] since routine measurements of this amine have proved to be extremely difficult due to its exceedingly low levels (down to 0.5 ng/g in rat brain [9, 10]). The levels of IAA are also low (10—15 ng/g in adult rat brain [9, 11]), but its determination does not pose the same difficulties as in the case of its parent amine. Two selected ion monitoring (SIM) methods have been developed recently for its quantification in brain tissue [9, 11]. Also, a high-performance liquid chromatographic (HPLC) procedure, involving a modified fluorimeter cell, has been described [12] and successfully applied [8].

Although SIM (isotope dilution technique) is considered to be a reference

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analytical technique, it has a number of disadvantages for routine work: the need of obtaining suitable chromatographic derivatives with high enough yield, the problem of adsorption and/or degradation of labile compounds [13], and the high cost of the equipment could be mentioned as the most important ones. In contrast, HPLC coupled to fluorimetric detection (HPLC—F) offers a remarkable degree of specificity (capitalizing on the natural fluorescence of indolic compounds) and less sample handling, although in the absence of modified equipment [12] the sensitivity of SIM is several times better than that achieved by HPLC—F.

This work describes a comparison of the SIM and HPLC—F techniques for the assay of IAA in rat brain tissue, using standard equipment for both methods.

## MATERIAL AND METHODS

### *Chemicals*

Standard indole-3-acetic acid was purchased from Sigma (St. Louis, MO, U.S.A.). Side-chain deuterated IAA (IAA-$d_2$) was from Merck, Sharp and Dohme (Montreal, Canada). Isotopic purity of IAA-$d_2$ was checked by SIM and was found to be better than 98%.

Pentafluoropropionic (PFP) anhydride (Regis, Morton Grove, IL, U.S.A.) was used to obtain the PFP derivatives.

All solvents and reagents were analytical grade and were used without further purification, except ethyl acetate and methanol which were distilled in an all-glass apparatus. Water used for the preparation of the homogenization media was distilled over potassium permanganate.

Stock solutions of IAA were prepared in distilled methanol, and stored at —30° C. Working solutions (approx. 1 ng/$\mu$l) were prepared by dilution for each experiment.

### *HPLC instrumentation*

The HPLC system consisted of a solvent delivery pump (Model 6000) and a U6K injector from Waters Associates (Milford, MA, U.S.A.). The column was a $\mu$Bondapak $C_{18}$ (particle size 10 $\mu$m). Column effluents were monitored with a 650-10S fluorimetric detector (Perkin-Elmer, Norwalk, CT, U.S.A.). Fluorescence excitation and emission wavelengths were set at 280 and 340 nm, respectively [14].

### *Gas chromatography—mass spectrometry (SIM) instrumentation*

A Hewlett-Packard 5995 gas chromatography—mass spectrometry (GC—MS) system was used, equipped with an open-split interface and a standard 9825A microprocessor. Samples were run on a 25 m × 0.3 mm I.D., bonded phase OV-101 capillary column, also from Hewlett-Packard (Palo Alto, CA, U.S.A.).

### *Experimental procedure*

*Homogenization and extraction.* Adult male Sprague-Dawley rats weighing 180—200 g were killed by decapitation, their brains removed and rapidly taken up in a plate on ice. The brain tissue was blotted with filter paper to eliminate

the contribution of blood IAA, weighed and homogenized. Homogenization was carried out in 10 volumes of 1 mol/l potassium chloride solution at pH 2.0 (adjusted with hydrochloric acid) containing 0.1% of ascorbic acid.

The homogenate was divided into sixteen aliquots taking eight of them for GC—MS and the other eight left for HPLC, as described below.

The corresponding homogenates were centrifuged in a J-21 Beckman high-speed centrifuge (50 000 *g*, 30 min) and the supernates were processed as follows. To each of them (in a capped glass test tube), 6 ml of freshly distilled ethyl acetate were added. After vigorous shaking for 5 min, they were centrifuged at 1000 *g* for 5 min. This procedure was repeated once and the pooled organic extracts were evaporated in a rotary film evaporator at 37°C using heart-shaped flasks, to a final volume of 200—400 μl. The extracts were next transferred to 1 ml Regis conical microvials, and pooled with the 2 × 200 μl of distilled methanol used to wash the walls of the heart-shaped flasks. The extracts were evaporated to dryness under a helium flow.

*Addition of standards for quantitative purposes.* The internal standard (IAA-$d_2$) used for the GC—MS (SIM) determinations and the supplements of reference IAA added for quantification in HPLC by the method of "spiking" were added to aliquots of the same homogenate in order to insure that both were subjected to the same experimental conditions as the endogenous brain IAA. Thus for GC—MS studies a total of eight aliquots (6 ml each, pipetted under gentle stirring) were all supplemented with 41 ng of IAA-$d_2$ and either 0, 11.5, 34.5 or 57.5 ng of unlabelled IAA (concentration corresponding to 0, 20.4, 61.2 and 102.0 ng/g, respectively) obtaining duplicate samples A, B, C and D. The same sample preparation procedure was used for the HPLC analysis, except that no labelled IAA was added, resulting in an equivalent "spiked" duplicate sample series for the quantitative HPLC determinations. All this is summarized in Table I.

TABLE I

SCHEME OF SAMPLE PREPARATION

| GC—MS | | | HPLC | | |
|---|---|---|---|---|---|
| Sample | ng IAA-$d_2$ | ng IAA | Sample | ng IAA-$d_2$ | ng IAA |
| $A_{GC}$ | 41 | 0 | $A_{LC}$ | 0 | 0 |
| $B_{GC}$ | 41 | 11.5 | $B_{LC}$ | 0 | 11.5 |
| $C_{GC}$ | 41 | 34.5 | $C_{LC}$ | 0 | 34.5 |
| $D_{GC}$ | 41 | 57.5 | $D_{LC}$ | 0 | 57.5 |

*HPLC procedure.* The HPLC identification and quantitative fluorimetric detection of IAA was carried out isocratically with 0.01 mol/l sodium acetate at pH 4.0 and 40% methanol. Eluents were filtered and degassed before use. The dry extracts were dissolved in 300 μl of the chromatographic eluent and aliquots of 30 μl were injected.

The content of IAA in each sample was calculated by the method of the added standard (sample supplemented with known amounts of IAA), which has proved to be useful in the determination of IAA in human plasma samples [15].

The precision of the HPLC method was evaluated by processing and analysing in duplicate six non-supplemented homogenates, corresponding to 0.52 g of brain tissue.

*GC—MS procedure.* Samples were chromatographed on an OV-101 bonded phase capillary column (25 m × 0.3 mm I.D.). Injection was carried out in the splitless mode. Helium was used as carrier gas. Injection port temperature was set at 250°C and injection column temperature at 60°C. Injection time was 0.7 min. Column temperature was programmed at 10°C between 170 and 250°C. The IAA-Me-1PFP derivative eluted from the column at 185°C approximately.

The dried samples for SIM analysis were treated according to described procedures [9] in order to obtain the methylpentafluoropropionyl derivatives of IAA and IAA-$d_2$ (IAA-Me-1PFP). For the methylation of the free carboxyl group, 20 $\mu$l of HCl—methanol (10%, v/v) were used instead of the $BCl_3$—methanol. The final solution was in 20 $\mu$l of isooctane and aliquots of 2—4 $\mu$l were injected into the GC—MS system.

The content of IAA in each sample was calculated by comparing the peak heights of the corresponding deuterated and non-deuterated compound.

The *m/z* values used to monitor the elution of IAA-Me-1PFP and its corresponding deuterated internal standard were 276 and 335 ($M^+$) for IAA and 278 and 337 ($M^+$) for IAA-$d_2$. Routine determinations were performed by comparing peak heights of either 278 vs. 276 or 337 vs. 335, the latter providing somewhat cleaner SIM profiles, even though their corresponding abundances in the mass spectra of IAA and IAA-$d_2$ are lower than those of 276 and 278.

The precision of the GC—MS method was evaluated by processing and analysing five identical samples containing 4.1 ng of IAA-$d_2$ (equivalent to 7.9 ng of IAA-$d_2$ per g of tissue).

## RESULTS AND DISCUSSION

The GC—MS technique is the most sensitive for the determination of low levels of IAA. Using capillary columns, 5 pg of authentic IAA can be unequivocally detected at a signal-to-noise ratio of 2. In contrast, the detection limit by HPLC can be set at 150 pg.

Fig. 1 shows the GC—MS (SIM) and HPLC profiles corresponding to a brain extract. In both cases, these profiles are free from interference. The identity of IAA in GC—MS is confirmed by monitoring also the ion at *m/z* 335 (molecular ion) and by the practical coincidence of the retention times corresponding to the peak of IAA (*m/z* 276 and 335) and the internal standard IAA-$d_2$ (*m/z* 278 and 337). In HPLC the identity is assigned by the increased response when the sample is supplemented with IAA as well as by the coincidence of retention times between standard IAA and brain IAA.

Fig. 2 shows the least-square roots correlation of quantitative data for the two methods in the range of 18—120 ng/g. Points represent the mean of duplicate (HPLC runs) or triplicate (SIM runs) determinations carried out on all of the duplicate homogenates. The corresponding parameters are [SIM value = $a + b$ (HPLC value)] $a$ = 0.8 ng/g, $b$ = 0.995, and $r^2$ = 0.991. Both parameters $a$ and $b$ indicate that the accuracy of each method is almost identical.

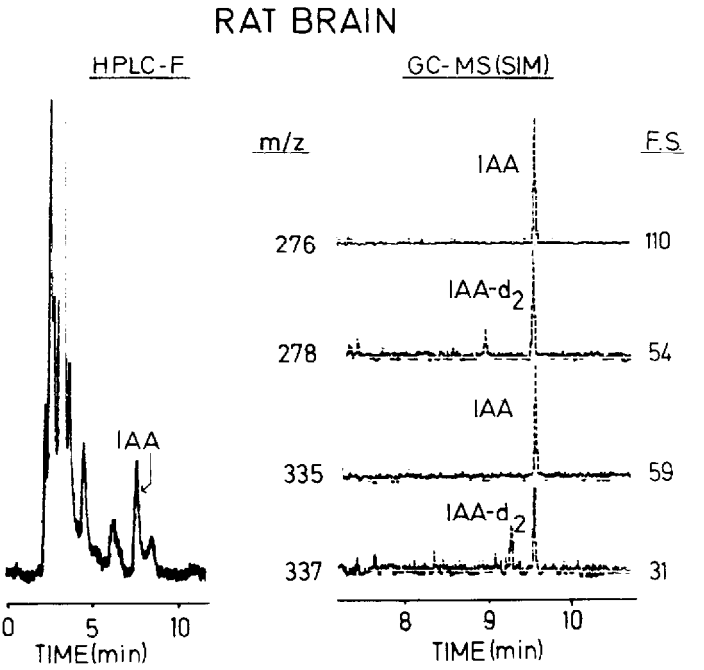


Fig. 1. HPLC—F and GC—MS (SIM) profiles corresponding to a brain extract. The figures on the right (F.S.) refer to the full-scale values of each of the GC—MS (SIM) traces.

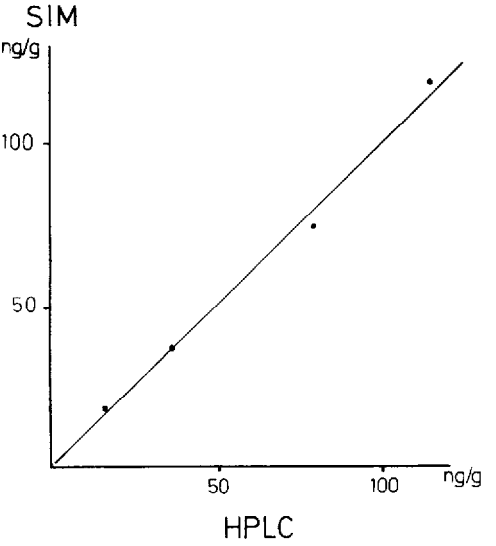


Fig. 2. Correlation of quantitative data for GC—MS (SIM) and HPLC methods. SIM value = $a + b$ (HPLC value), where $a = 0.8$, $b = 0.995$, $r^2 = 0.991$.

The coefficients of variation were 7.1% for HPLC and 8.5% for SIM. Such a result could be considered somewhat surprising and unexpected in view of the high precision usually claimed for the selected ion monitoring methods in GC—MS. However, it could be readily accounted for by the following practical facts. When the GC column was programmed at a rate of 10°C/min, the

coefficient of variation was as high as 17%. Under these conditions, and using the minimum dwell time for the two monitored *m/z* values (335 and 337), the peak profile of IAA-Me-1PFP consisted of only 5—6 sampling points. The entire peak width was less than 3 sec, so that the reliability of any measurement of peak maxima could be relatively poor. In contrast, it must be noted that the reported SIM precision has been obtained using a low program rate (2° C/min), so that, under these conditions, the chromatographic peak profile of IAA-Me-1PFP consists of at least ten steps, thus achieving a better definition of the true peak maxima. Consequently, a reduced peak width, as obtained with capillary columns, is not always an advantage for quantitative precision work in the GC—MS (SIM) mode. It must be taken into account that the coefficient of variation in GC—MS (SIM) is free from errors due to injection, derivatization and overall sample loss since it is calculated relative to deuterated analog used as internal standard, which does not apply in the case of HPLC runs. The fact that the precision in the GC—MS (SIM) mode is somewhat poorer must reflect the practical limitation of this and similar instrument software for fast ion switching within the short time domain of a peak eluted from a capillary column. In accordance, to increase the reliability of peak height measurements, we have found it advisable to degrade the GC performance by going to longer elution times, thus obtaining wider peaks.

In summary, we have shown that the two methods herein described, based on a classic extraction and further analysis by HPLC or GC—MS (SIM), give practically identical accuracy and precision for the assay of IAA in brain tissue. However, HPLC would be considered in this case as the method of choice for routine purposes, while GC—MS (SIM) would be very useful in cases requiring a higher sensitivity or for confirmatory purposes.

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