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EVALUATION OF THE POTENTIAL OF THERMOSPRAY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY IN NEUROCHEMISTRY

F. ARTIGAS and E. GELPÍ*
Departamento de Neuroquímica, CSIC, Jorge Girona Salgado, 18-26, 08034 Barcelona (Spain)

SUMMARY

Optimized operating conditions previously developed for the determination of neuroactive indoleamines and metabolites were adapted to meet the requirements of thermospray liquid chromatography–mass spectrometry (LC–MS) in terms of the ammonium acetate buffer system needed in this technique. Mass spectra were obtained for nineteen indolic compounds in both the positive and negative ion modes.

The positive thermospray mass spectra of indoles with a free primary amino group are characterized by the base peak at $[M + H]^+$, whereas the alcohol and acid metabolites show the base peak at $[M + NH_4]^+$. In the negative mode only amino acids and acids give good mass spectra with base peaks at $[M - H + ACOOH]^-$. Detection limits by selected ion monitoring were of the order of 50–100 pg SIM on-column, allowing the direct determination of endogenous serotonin in an extract from rat hypothalamus. Quantitation was performed by isotope dilution MS. In the same way 5-hydroxyindoleacetic, indoleacetic, indolepropionic and indolelactic acids in urine were directly determined in an ethyl acetate extract from acidified urine samples. Likewise, γ-aminobutyric acid and tricyclic antidepressants gave detection limits of 10 pg whereas only nanogram sensitivity could be achieved with catecholamines.

INTRODUCTION

With the advent of reversed-phase columns and specific electrochemical and fluorescence detectors, high-performance liquid chromatography (HPLC) has become one of the major tools in neurochemical research[1]. Nevertheless, it still lacks the identification power and selectivity of mass spectrometry (MS), a technique which itself has contributed greatly to neurotransmitter determinations[2]. The mass spectrometer as a chromatographic detector is sensitive, specific and certainly more universal than electrochemical or fluorescence HPLC detectors. However, until recently only the coupling of MS with gas chromatography (GC–MS) had achieved the level of technological feasibility needed for everyday routine operation, although subject to practical limitations as regards the direct assay of complex biological extracts. On the other hand, within the last 5 years it has become evident that HPLC–MS coupling could soon also constitute a practical method.

The commercial introduction of interfaces suitable for HPLC–MS operation

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has contributed to the generation of interest in the potential applications of this technique[3]. Of the three main types of HPLC–MS interfaces available [moving belt, direct liquid introduction and thermospray (TSP)], the last seems at present to be the most promising and several applications have already been described in fields such as the analysis of environmental samples and pesticides[4,5], eicosanoids[6,7] and phospholipids[8]. Other applications, not only of TSP but also of other interfaces, can be found in the Proceedings of the previous LC–MS Symposium in 1984[9] and the 10th International MS Conference[10]. Nevertheless, despite the growing interest in the TSP HPLC–MS technique, hardly any applications in the field of neurochemistry have been described. Hence the aim of this work was to evaluate the potential of the method in different situations involving compounds of neurochemical interest, especially the neuroactive metabolites of tryptophan and related indoles and also γ-aminobutyric acid (GABA), tricyclic antidepressants and 3-methoxy-4-hydroxyphenylethylene glycol (MHPG).

EXPERIMENTAL

Chemicals and reagents

Acetic acid and ammonia solution (analytical-reagent grade) for the preparation of 0.1 mol/l ammonium acetate solution were obtained from Merck (Darmstadt, F.R.G.). Distilled water was obtained with an all-glass apparatus. Formic acid (Merck) was used to adjust the pH of HPLC buffers. Methanol (HPLC grade) was purchased from Scharlau (Barcelona, Spain). Standards of neurochemical compounds (indoleamines, catecholamines, GABA, antidepressants and MHPG were from commercial sources. Polyethylene glycol (PEG) (average MW 600), used for the mass axis calibration, was a gift from Dr. V. Raverdino (Hewlett-Packard, Geneva, Switzerland). Other chemicals used in the sample treatment (perchloric acid, ethyl acetate, butanol, etc.) were analytical-reagent grade from local suppliers.

Sample preparation and extraction procedures

Indole acids in urine. A 0.5-ml volume of urine from a healthy individual was adjusted to pH 2.0 with hydrochloric acid and 2 ml of ethyl acetate were added. The mixture was vigorously shaken for 5 min, and after centrifugation, the organic layer was placed in a freezer (−20°C) for 10 min to eliminate the remaining water. Then it was centrifuged again and transferred into a conical-bottomed flask where it was evaporated to dryness. A 200-μl volume of 0.1 *M* ammonium acetate solution was added and aliquots were injected into the HPLC–MS system.

Serotonin (5HT) and GABA in brain tissue. Regions of brain tissue were dissected out on a plate on ice and homogenized in 0.4 *M* perchloric acid. The homogenate was centrifuged (10000 *g*, 20 min) and the supernatants were taken for analysis. 5HT was extracted from homogenates using a modified *n*-butanol procedure[11] which involves the extraction of 5HT into *n*-butanol and addition of *n*-heptane and few drops of 0.1 *M* hydrochloric acid to back-extract the amine into the aqueous phase, which was injected into the HPLC–MS system. GABA was analysed by direct injection of brain homogenates.

HPLC–MS parameters

All applications described here were carried out on a 5988 A mass spectrometer equipped with a thermospray interface, an ion source for direct HPLC–MS and a desk-top work-station, equipped with plotter facilities (all from Hewlett-Packard, Palo Alto, CA, U.S.A.). The HPLC system connected to the inlet of the thermospray interface consisted on two Model 6000 A pumps, a Model 600 programmer and a U6K injector (all from Waters Assoc., Milford, MA, U.S.A.). As required for optimum performance of the TSP system, the HPLC eluent was 0.1 mol/l ammonium acetate buffer with different proportions of methanol in either isocratic or programmed operation, as indicated. For all compounds tested, the sensitivity was found to be much better when methanol was used instead of acetonitrile as an organic modifier. The eluent flow-rate was maintained at 1 ml/min. The HPLC columns used were 5 μm Spherisorb ODS-1 (15 cm × 4.6 mm I.D.) (Tracer Analítica, Barcelona, Spain).

The outlet of the columns was connected to the inlet of the TSP interface through a short (*ca.* 30 cm) narrow-bore stainless-steel tube. Mass axis (m/z) calibration was performed using the Manual Tune program, focusing on those positive or negative ions (depending on the mode of operation) arising either from the buffer (low m/z), imipramine (medium m/z) and polyethylene glycol (high m/z). The polyethylene glycol used for calibration was usually kept to a minimum (it was adjusted to give a signal less than 5% of that of the major ions in plasma), because after several days of heavy work the HPLC connections became slightly contaminated, giving a constant background signal corresponding to PEG ions.

Calibration was performed so that maximum sensitivity was obtained in the mass range of the ions expected.

The temperature of the different zones of the TSP interface and ion source were adjusted within the following ranges, depending on the type of sample and HPLC elution conditions selected: vaporizer probe stem, 104–116°C; probe tip, 184–204°C; vapour, 200–210°C; and source block, 270°C. The optimal temperature of the stem vaporizer probe was adjusted with reference to a plot of vapor temperature *vs.* stem temperature. Usually, the stem temperature was adjusted so that the extent of vaporization of the buffer was 95%. When working in the gradient mode, the stem temperature was calculated for both the initial and final eluent compositions and an inverse gradient stem temperature was applied when running the samples.

Specific settings for each application are indicated in each instance. Occasionally, when working with authentic standards direct flow injection analysis was performed by connection of the HPLC injector to the inlet of the TSP probe, bypassing the column in this instance.

RESULTS AND DISCUSSION

TSP HPLC–MS of neuroactive indoles

As there are no data available on the thermospray spectra of neuroactive indoles, a study was undertaken to characterize the behaviour of a number of these compounds by positive and negative TSP ionization using filament on and off. Table I summarizes the most significant peaks and corresponding abundances for each of the nineteen indoles included in this study.

TABLE I

POSITIVE THERMOSPRAY MASS SPECTRA OF INDOLES

*Compound**	*Ions observed, m/z (%)***				
	$(M + H)^+$	$(M + NH_4)^+$	$(M + H + 32)^+$	$(M + H + 64)^+$	*Other*
TP	205 (100)	222 (3)	237 (32)	269 (2)	
5HTP	221 (100)	238 (7)	253 (28)	285 (2)	205 (33) 237 (12)
5MeOTP	235 (100)	252 (9)	267 (22)	299 (2)	295 (3)
T	161 (100)	179 (4)	193 (41)	225 (4)	321 (43)
5HT	177 (100)	194 (1)	209 (15)	241 (1)	353 (2)
5MeOT	191 (100)	209 (10)	223 (42)	255 (3)	
N-wMeT	175 (100)	192 (1)	207 (23)	239 (1)	349 (2)
5MeODMT	219 (100)		251 (1)		
Bufotenine	205 (100)		236 (3)		
THBC	173 (66)	191 (6)	205 (100)	237 (18)	
N-Acetyl-5HT	219 (50)	236 (100)	251 (5)		268 (4)
			$(M + NH_4 + 32)^+$	$(M + NH_4 + 64)^+$	
Melatonin	233 (36)	250 (100)	282 (2)		
5HTOL	178 (61)	195 (100)	227 (5)	259 (2)	285 (7)
5MeOTOL	192 (85)	209 (100)	241 (8)		
IAA	176 (17)	193 (100)	225 (28)		
IPyA	203 (10)	221 (100)	253 (20)		
IPrA	190 (37)	207 (100)	239 (17)		396 (3)
5HIAA	192 (8)	209 (100)	241 (70)		
ILA	206 (12)	223 (100)	255 (10)		

* Abbreviations: TP, tryptophan; 5HTP, 5-hydroxytryptophan; 5MeOTP, 5-methoxytryptophan; T, tryptamine; 5HT, 5-hydroxytryptamine (serotonin); 5MeOT, 5-methoxytryptamine; N-wMeT, N-methyltryptamine; 5MeODMT, 5-methoxydimethyltryptamine; 5HTOL, 5-hydroxytryptophol; 5MeOTOL, 5-methoxytryptophol; IAA, indoleacetic acid; IPyA indolepyruvic acid; IPrA, indolepropionic acid; 5HIAA, 5-hydroxyindoleacetic acid; ILA, indoleactic acid; THBC, tetrahydro-β-carboline.

** The notation +32 and +64 refers to one and two molecules of methanol, respectively.

As indicated by the results, all of these compounds add hydrogen, $[M + H]^+$, ammonium $[M + NH_4]^+$ or different combinations of hydrogen, ammonium and methanol $[M + H + CH_3OH]^+$, $[M + H + 2CH_3OH]^+$, $[M + NH_4 + CH_3OH]^+$ and $[M + NH_4 + 2CH_3OH]^+$. Also, depending on the type of predominating adduct species, these spectra can be classified into two major groups. All indoles with amino acid or primary amine substituents at position 3 on the indole nucleus show base peaks corresponding to the $[M + H]^+$ adducts. On the other hand, when the substituent at position 3 is an alcohol, acid or N-acetylethylamine group (melatonin and N-acetylserotonin), the base peak corresponds to the $[M + NH_4]^+$ adduct. In a few instances there are ions with *m/z* values corresponding to the attachment of two molecules of analyte plus hydrogen $[2M + H]^+$, *e.g.*, *m/z* 321 for tryptamine and *m/z* 353 for serotonin.

As regards the predominance of the protonated $[M + H]^+$ or ammoniated $[M + NH_4]^+$ ions, it has been pointed out[5] that TSP ionization would be analogous

TABLE II

NEGATIVE THERMOSPRAY MASS SPECTRA OF INDOLES

Compound	*Ions observed, m/z (%)*			
	$(M - H)^-$	$(M + CH_3COOH - H)^-$	$(M + 2CH_3COOH - H)^-$	$(2M - H)^-$
TP	203 (28)	263 (100)		
5HTP		279 (100)		
5MeOTP	233 (36)	293 (100)	353 (4)	
ILA	204 (100)	264 (86)		409 (54)
IPrA	188 (6)	248 (60)	308 (5)	377 (100)
5HIAA	190 (10)	250 (100)	310 (4)	381 (40)
IAA	174 (23)	234 (95)	294 (6)	349 (100)

to ammonia CI, the ionization of the analyte depending on the relative proton affinities of analyte and buffer. Thus, ammonium acetate would behave as a sufficiently weak base to protonate relatively more basic indoles, whereas for the acid- or alcohol-substituted indoles their proton affinities would be lower than that of ammonium acetate. It has been proposed and experimentally supported[12] that gas-phase acid–base reactivity may account for the ionization of solutes in TSP HPLC–MS.

The negative TSP mass spectra of those compounds producing ions in this mode of operation are summarized in Table II. In this instance, no clear classification can be proposed on account of the type of adduct ions formed, as in the positive ion spectra. These mass spectral patterns are characterized by the $[M - H]^-$ ions and the ions arising from attachment of an acetic acid moiety to the $[M - H]^-$. In contrast to what is observed in the positive mode, ions corresponding in *m/z* value to $[2M - H]^-$ can be very abundant, as shown in Table II. Only those indoles with carboxylic groups give negative mass spectra in the low microgram range, as would be expected considering their relative proton affinities in a methanol–ammonium

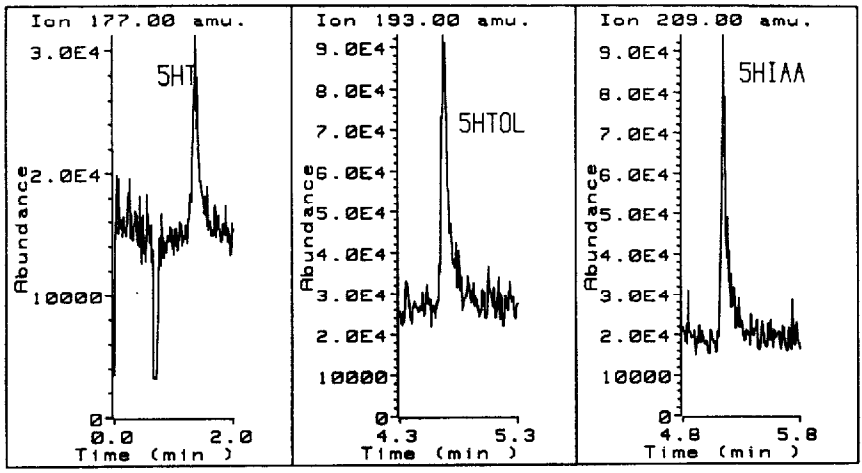

Fig. 1. Selected ion responses obtained by monitoring ions at *m/z* 177, 193 and 209 for 5HT (470 pg), 5HTOL (280 pg) and 5HIAA (380 pg), respectively. Eluent: ammonium acetate–methanol (40:60).

acetate medium. It should be noted that these spectra (Tables I and II) were obtained by direct injection of appropriate solutions of authentic standards on to a stream of HPLC mobile phase, flowing directly into the TSP probe. This would be analogous to performing flow injection analysis (FIA) using the mass spectrometer as an analyte detector and can be very useful for the rapid determination of the mass spectral patterns from a given compound or family of compounds as in this application. Thus, the TSP mass spectra of the nineteen indoles were obtained (in 14 min) by sequential injection of each compound on to a methanol–ammonium acetate (60:40) mixture flowing at 1 ml/min. This allows very simple and rapid screening of different analytes in order to establish a mass spectral data base for subsequent identification by computer matching procedures. Whenever the mass spectra of the analytes are not known beforehand this mode of operation can save analysis time.

All indole TSP mass spectra, in both the positive and negative modes, were obtained with the filament on as it was determined that the response could be increased 3.5-fold in this way relative to the filament-off operation. Under these conditions the detection limits at a signal-to-noise ratio of 2 varied from 50 to 100 pg. For instance, the TSP HPLC selected ion traces shown in Fig. 1 illustrate the responses obtained from injections of 5HT (470 pg), 5HTOL (280 pg) and 5HIAA (380 pg).

The reproducibility was found to be satisfactory in general as demonstrated by the 7.2% coefficient of variation ($\bar{x}$/S.D.) of the ion at m/z 161 for ten repetitive injections of 2 ng of T. Nevertheless, the variations in TSP systems can be important

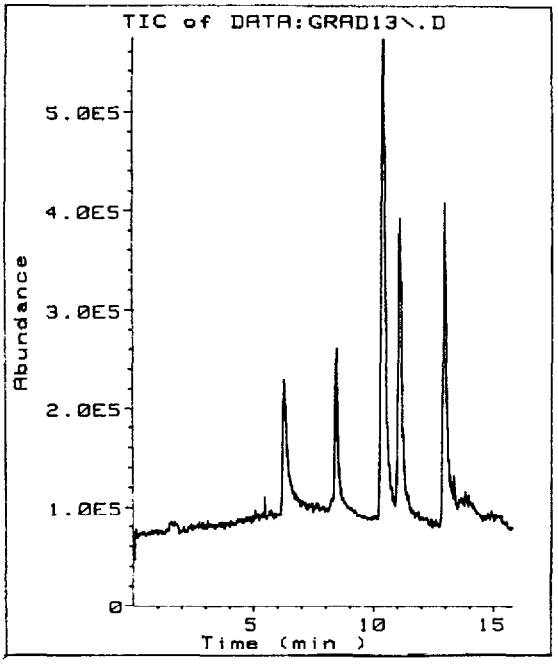

Fig. 2. Total ion current profile from a sample of standards (5HT, 5HIAA, T, 5MeOT and IAA, in order of elution) obtained by TSP HPLC–MS. The reversed-phase column was eluted with a 10-min 10–50% methanol gradient in 0.1 *M* ammonium acetate buffer (pH 4.0, adjusted with formic acid).

in some instances so that reliable quantitations should preferably be made with labelled internal standards. A plot of amount injected *vs.* response for the *m/z* 161 ion of T gave a straight line ($r = 0.9989$) in the 4–20 ng range. In this sense, quantitative performance of the TSP ion source for standard indoles is similar to that obtained in a conventional GC–MS electron impact ion source for these compounds[13]. One of the operative limitations of the TSP ionization lies in the need to use only volatile buffers to avoid deposits of inorganic salts in the ion source or even occlusion of the TSP capillary probe. Of all possible volatile organic buffers, ammonium acetate seems to be the most suitable in terms of protonating capabilities, intensity and reproducibility of response for different analytes[5].

However, existing methods for neurochemical studies of indoles by HPLC[1] commonly involve the use of eluents prepared using methanol, water, ion-pairing reagents and phosphate or citrate buffers. Accordingly, the analysis of these indoles by TSP HPLC–MS required a change from our previously optimized HPLC methods[11,14] to an eluent system based on the use of ammonium acetate buffer. The results obtained have demonstrated that the use of this buffer does not represent a chromatographic limitation for the HPLC separation of brain indoles, as illustrated in Fig. 2. Fig. 2 shows the separation achieved in less than 15 min for 5HT, 5HIAA, T, 5MeOT and IAA under gradient conditions. Thus, gradient elution is a definite possibility in TSP operation, provided that appropriate inverse interface temperature programming is carried out in parallel with eluent programming. This is necessary to compensate for changes in optimal initial temperature conditions due to the changing water content in the eluent.

Indole acids in human urine

Aliquots of urine samples, extracted as indicated under Experimental, were injected and eluted under the same conditions as in Fig. 2. The responses obtained

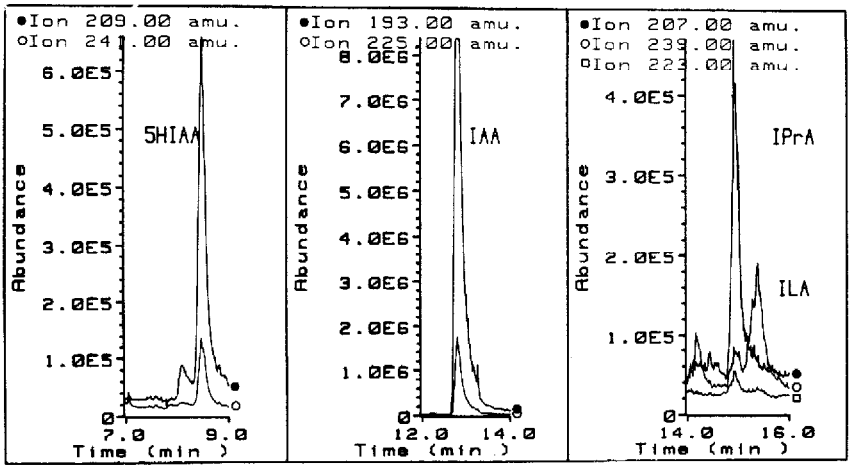

Fig. 3. Selected ion current profiles corresponding to indole acids in a sample of human urine. Gradient: 10–50% methanol in 0.1 *M* ammonium acetate (pH 4.0). The ion profiles show the presence of four different indole acids, as indicated by their respective retention times and the response at characteristic *m/z* values (see Table I).

by selected ion monitoring (SIM) of the appropriate ions, as shown in Fig. 3, allow the determination of 5HIAA, IAA, IPrA and ILA in urine samples.

Serotonin in rat brain tissue

The determination of serotonin in homogenates of rat hypothalamus by TSP HPLC–MS under SIM conditions is shown in Fig. 4. Elution was carried out as in Fig. 2. For quantitative purposes labelled serotonin[13] was used as an internal standard, as shown by the lower trace in Fig. 4. The use of SIM is essential in this instances for sensitivity considerations as endogenous 5HT is present in rat hypothalamus in the low nanomoles per gram range (3.86 nmol/g in this particular sample). As with acids in urine, mentioned above, a conventional GC–MS determination of this compound would imply a more complex experimental procedure as samples would need to be properly dried and derivatized[13]. Most indolic compounds are relatively stable in the low nanogram range during the extraction and derivatization steps needed for their GC–MS analysis. However, others, especially those with more polar groups (*e.g.*, 5HTP and IPyA), are prone to decompose easily, resulting in an overall decrease in sensitivity. Moreover, when working with small amounts (picogram–nanogram range) of the "stabler" indoles, the apparent stability is partly lost owing to irreversible adsorption, thermal degradation or lack of proper derivatization during the experimental procedure[15]. These considerations, in addition to its relative ease of use, have made HPLC the technique of choice for the determination of small amounts of indolic compounds.

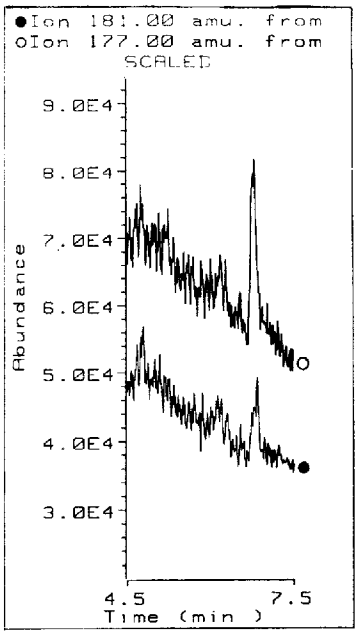

Fig. 4. Ion profiles at *m/z* 177 (upper trace) and *m/z* 181 (lower trace) corresponding to brain (hypothalamus) 5HT and deuterated 5HT, respectively. Gradient elution was performed as in Fig. 3.

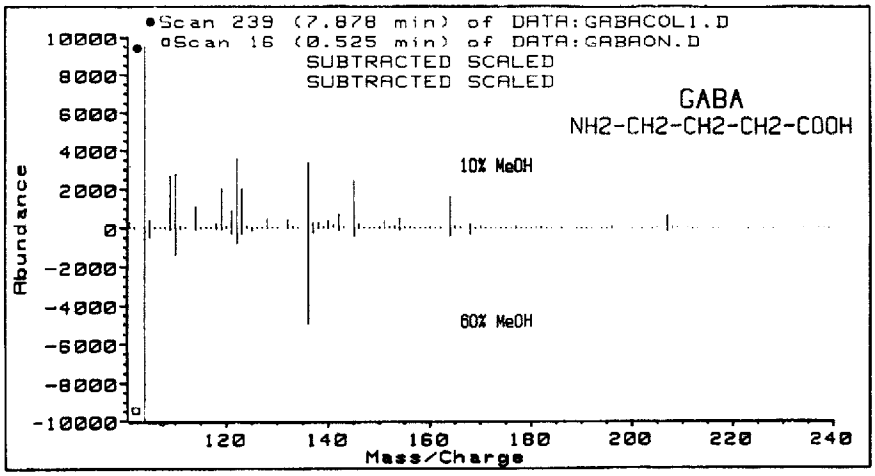

Fig. 5. Positive TSP mass spectra of GABA using different methanol concentrations in the eluent.

Determination of GABA in rat brain tissue

The TSP spectra of GABA obtained with different methanol concentrations in the HPLC eluent are shown in Fig. 5. An evaluation of the two spectra indicates that the addition of ammonia is more favourable at a lower methanol content (see the ion at *m/z* 122, corresponding to $[M + H]^+$ at *m/z* 104 plus 18 a.m.u.). On the other hand, at 60% methanol the $[M + NH_4]^+$ ion is relatively much less abundant than the $[M + H + CH_3OH]^+$ ion at *m/z* 136. In both instances, a detectable ion at *m/z* 207, (corresponding to $[2M + H]^+$) is observed. Owing to its amphoteric properties, the GABA molecule has a net charge. This, together with its low molecular weight, is the cause of the poor retention of GABA by the reversed-phase column used (Spherisorb ODS-1). For this reason, it elutes shortly after the injection front, regardless of the pH of the ammonium acetate buffer or the eluotropic strength of the eluent. This could clearly be a disadvantage when using conventional HPLC detectors (*e.g.*, UV detection)[16] because the GABA signal could be masked by other co-eluting compounds in the chromatographic front. However, this is not the case in HPLC–MS where SIM allows for a specific, independent detection of GABA. Moreover, its short retention time provides an extra degree of sensitivity because of the enhancement of the signal-to-noise ratio owing to the narrow amplitude of the GABA chromatographic peak (10–12 s, see Fig. 6). Under these conditions the limit of detection obtained by SIM at *m/z* 104 can be set at 10 pg, which is comparable to or even better than the sensitivity achieved by GC–MS. Fig. 6 shows the SIM profile at *m/z* 104 corresponding to a direct injection (2-μl aliquot) of perchloric acid brain homogenate (hippocampus). As with 5HT in the hypothalamus, an appropriate quantitation of GABA in brain tissue would require the use of deuterated GABA as an internal standard (not available in the laboratory when this study was performed). Under the present experimental conditions, the GABA detected corresponds to a sample of brain tissue weighing 200 μg. This, on account of the extremely favourable conditions for the assay of this compound (high sensitivity, short analysis time, ease of sample handling), opens the way to study the GABA neurochemistry of very limited brain areas by HPLC–MS.

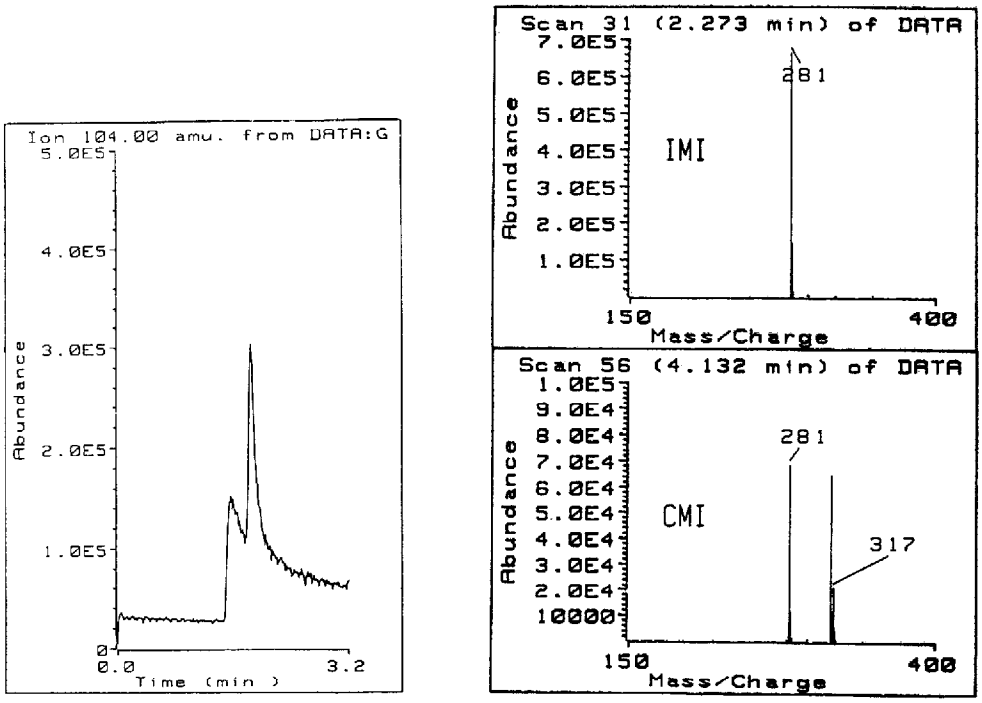

Fig. 6. Selected ion profile at m/z 104 $[M + H]^+$ for endogenous GABA from a sample of brain homogenate (hippocampus). Isocratic elution with ammonium acetate (pH 3.35)–methanol (90:10).

Fig. 7. Positive TSP mass spectra of the tricyclic antidepressants, imipramine and chlomipramine. The spectra were obtained by direct injection into the TSP interface. Mobile phase: ammonium acetate–methanol (40:60).

Other compounds of neurochemical interest

The TSP mass spectra of two tricyclic antidepressants, imipramine (IMI) and chlomipramine (CMI), are depicted in Fig. 7. The $[M + H]^+$ ion in the imipramine spectrum (m/z 281) is displaced to 335 by the presence of chlorine in the CMI spectrum. The fragment ion arising from chlorine loss in the CMI spectrum should be at m/z 280 but it also appears at m/z 281, indicating protonation. The limit of detection for IMI has been set at 10 pg, which, again, turned out to be better than that obtained with GC–MS methods. Regarding detection limits, it has been observed that in TSP operation these are particularly sensitive to the relative concentration of other components in biological samples co-eluting with the compound of interest. In other words, the abundance of the $[M + H]^+$ ions of the analyte, for instance, would depend on the extent of competing protonation or molecular association reactions proceeding concomitantly in the ion source. Thus, even though HPLC–MS in principle has the advantage over GC–MS of requiring less sample pre-treatment so that even direct assays of tissue homogenates can often be performed, appropriate sample clean-up procedures must occasionally be used to reduce background interferences whenever sensitivity becomes a limiting factor.

Also, an interesting observation concerning the high-mass attachment ions of the type $[2M + H]^+$ is their strong dependence on a minimum threshold concentration of the parent molecular species so that there is a clear differential behaviour in the selected ion traces of these ions *versus* those ions arising by first-order protonation or NH_4^+ attachment processes. In other words, the abundance profiles of high-mass $[2M + H]^+$ attachment ions show a steep downward slope with almost no tail, whereas the superimposed $[M + H]^+$ or $[M + NH_4]^+$ ions from the same molecular species are affected by tailing effects.

As shown above, the TSP ion source can be useful for the determination of indoles at the picogram level. In contrast, the response for catechols was found to be poor in general, with either methanol or acetonitrile as organic modifier. The spectra obtained showed the same patterns of production of ions as those observed for indoles ($[M + H]^+$, $[M + NH_4]^+$, $[M + H + CH_3OH]^+$, etc.), although to a lesser extent. The best detection limit observed was for MHPG (*ca.* 2 ng). This was achieved by focusing on the $[M + NH_4]^+$ ion at *m/z* 202 which is the base peak under the conditions used [ammonium acetate–methanol (20:80)]. Therefore, unlike other neurotransmitters such as the indoleamines or GABA, catecholamines and related metabolites seem to lack the necessary sensitivity in HPLC–MS for useful neurochemical studies where a high degree of sensitivity is usually needed. In conclusion, this work demonstrates the feasibility of using TSP HPLC–MS for the determination of a wide range of compounds of neurochemical interest in biological samples. Especially remarkable is the possibility of the direct assay of extracts or homogenates, in contrast to GC–MS where derivatization is mandatory in most instances. Also, the sensitivity seems to be acceptable for these compounds, being similar to that obtained in GC–MS, for example for authentic indoles, or even better, as with GABA. However, this may not hold true when analysing the same compounds in complex biological matrices owing to the ionization "quenching" effect mentioned above.

This approach should facilitate specific searches for a given compound or group of compounds in biological screening procedures especially whenever there is no prior information as to the type of functional groups present inasmuch as the uncertainties inherent in the derivatization procedure are eliminated.

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