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Short Communication

Applicability of high-performance liquid chromatography–continuous-flow fast atom bombardment mass spectrometry for simultaneous quantitation of multiple neurochemicals

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ABSTRACT

A method is described for the analysis of multiple neurochemically important compounds. The technique involves high-performance liquid chromatographic separation in combination with ultraviolet and continuous-flow fast atom bombardment mass spectrometric detection. High-performance liquid chromatographic elution behaviour and relative detection responsiveness as correlated with chemical structure are also presented.

INTRODUCTION

The complexity of the interactions which take place between various neurochemical systems during behavioral, pharmacological or psychological investigations requires that a comprehensive analytical strategy be used in order to study these phenomena. In one approach to this problem, simultaneous quantitative determination of multiple neurochemicals is utilized to provide a profile of the changes which are occurring in the central nervous system. Instrumental techniques such as gas chromatography (GC), GC–mass spectrometry (GC–MS) and high-performance liquid chromatography (HPLC) have been widely used for this purpose. In particular, recent developments in HPLC technology make it possible to separate and quantitate numerous organic compounds without requiring extensive sample preparation or chemical derivatization. Moreover, the combination of HPLC with MS permits the analysis of polar, non-volatile or thermally labile substances which are not amenable to GC–MS techniques. As an alternative to thermospray [1,2] or moving-belt [3–5] interfaces, we utilize direct sample introduction into an HPLC–continuous-flow fast atom bombardment (CF-FAB)-MS system [6,7] to accomplish these measurements. We have recently reported on our use of this method for quantitative analysis of acetylcholine (ACh) in rat brain regions [8].

In the present report we describe our results for analysis of 29 standard neurochemicals which have been evaluated for their LC elution behavior and suitability for quantitation by HPLC–CF-FAB MS. Furthermore, we show correlations between individual chemical structure and sensitivity by this technique.

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EXPERIMENTAL

Chemicals

A list of the standard compounds used in this investigation is presented in Table I. DOPAC, nMET and DOMA were purchased from Aldrich (Milwaukee, WI, USA); all of the other standards were obtained from Sigma (St. Louis, MO, USA). HPLC solvents and other reagents were of the highest purity available from commercial sources.

Continuous-flow FAB LC–MS system

A block diagram of the HPLC–CF-FAB-MS system is shown in Fig. 1. (Note that the manufacturer, JEOL, uses the term "FRIT-FAB LC–MS" for this technique.) Details about this system were recently presented by Ikarashi *et al.* [8]. HPLC separation was accomplished with an octadecyl silane semi-micro column (SFPAC-ODS05 S15 column, 15 cm × 1.7 mm I.D., 5 μm particle size, JEOL, Tokyo, Japan). Binary gradient elution was performed with mobile phase A comprised of 0.2% (v/v) trifluoroacetic acid (TFA) in water containing 1% glycerol, and mobile phase B consisting of 0.2% TFA in methanol containing 1% glycerol. The separation was initiated with 100% A, and the content of B was linearly increased from 0 to 40% over a period of 15 min. The flow-rate was maintained at 0.15 ml/min. After passing through the UV detector, the effluent from the column was mixed with a 1%

TABLE I

HPLC RETENTION TIME OF SELECTED NEUROCHEMICALS USING UV AND MASS SPECTROMETRIC DETECTION

Compound	Abbreviation	Mol. wt.	Retention time (min)[a]	
			UV	MS
Choline	Ch	104	n.d.	3.6 ± 0.2
Norepinephrine	NE	169	2.4 ± 0.1	3.7 ± 0.5
Octopamine	OCT	153	3.2 ± 0.1	4.4 ± 0.4
3,4-Dihydroxymandelic acid	DOMA	184	3.4 ± 0.1	n.d.
Epinephrine	EP	183	3.7 ± 0.2	4.9 ± 0.4
Ethylhomocholine	EHC	132	n.d.	5.1 ± 0.2
3,4-Dihydroxybenzylamine	DHBA	139	4.2 ± 0.2	5.5 ± 0.4
Acetylcholine	ACh	146	n.d.	5.7 ± 0.3
Normetanephrine	NMN	183	4.7 ± 0.2	5.9 ± 0.4
Synephrine	SYN	167	5.3 ± 0.2	6.7 ± 0.4
Dopamine	DA	153	5.8 ± 0.2	7.2 ± 0.4
Metanephrine	MN	197	6.4 ± 0.5	8.1 ± 0.6
Deoxyepinephrine	DEP	167	6.5 ± 0.5	8.1 ± 0.5
3,4-Dihydroxyphenylalanine	DOPA	197	7.0 ± 0.5	8.5 ± 0.5
Tyramine	TYM	137	7.0 ± 0.4	8.5 ± 0.5
Phenylethanolamine	PEOHA	137	n.d.	8.9 ± 0.4
Tyrosine	TYR	181	8.7 ± 0.2	10.0 ± 0.4
3-Methoxytyramine	3-MT	167	8.7 ± 0.2	10.0 ± 0.5
5-Hydroxytryptamine	5-HT	176	8.9 ± 0.2	10.3 ± 0.4
N-Methyl-5-hydroxytryptamine	nMET	190	9.3 ± 0.3	10.7 ± 0.5
Homovanilic acid	HVA	182	10.0 ± 0.3	11.5 ± 0.4
5-Hydroxytryptophan	5-HTP	220	10.4 ± 0.3	11.7 ± 0.3
Phenylalanine	PHE	165	n.d.	12.1 ± 0.2
Phenylethylamine	PEA	121	n.d.	12.3 ± 0.2
5-Hydroxytryptophol	5-HTOL	177	11.0 ± 0.4	12.6 ± 0.4
3,4-Dihydroxyphenylacetic acid	DOPAC	168	11.9 ± 0.5	13.2 ± 0.4
5-Hydroxyindoleacetic acid	5-HIAA	191	13.4 ± 0.2	14.8 ± 0.4
Tryptamine	TM	160	13.5 ± 0.2	14.8 ± 0.3
Tryptophan	TRP	204	14.1 ± 0.2	15.4 ± 0.5

[a] n.d. = not detected; values represent the mean ± standard deviation for five separate injections.

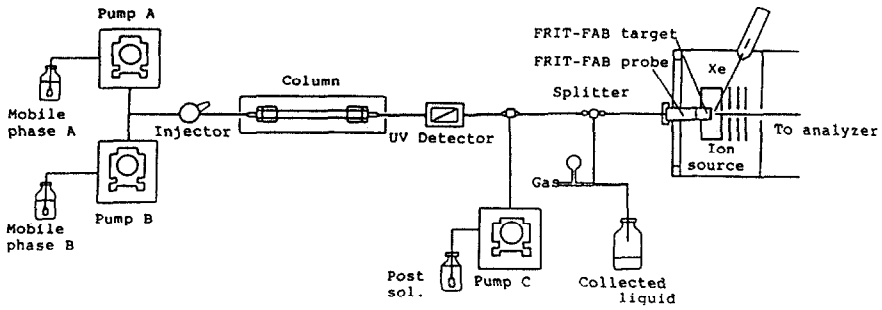


Fig. 1. Block diagram of the HPLC–CF-FA-MS system. For more details about the instrumentation, see ref. 8.

glycerol–methanol solution and introduced into the interface region (the splitter, connector and CF-FAB probe) at a final flow-rate of 0.25 ml/min, the maximum flow-rate which could be readily accomodated by the vacuum system. Approximately 8 μl/min of the effluent were transmitted into the mass spectrometer, and the remainder was collected. Mass spectra were acquired on a JEOL Model JMS-AX505W mass spectrometer in combination with a JEOL Model JMA-OA 5000 data system which was used for mass spectra data processing. MS conditions were as follows: ion source temperature, 50°C; accelerating voltage, 3 kV; conversion dynode voltage, −10 kV; electron multiplier voltage, 1.2 kV; resolution, 1000. A liquid nitrogen trap in the ion source region was used to improve the speed of evaporation. The mass spectrometer was either scanned from 30 to 800 a.m.u. (positive-ion detection) for qualitative analyses or operated in the selected-ion monitoring (SIM) mode for quantitative determinations. A JEOL atom gun was used with xenon (5 keV) for FAB from the surface of the flow FAB probe.

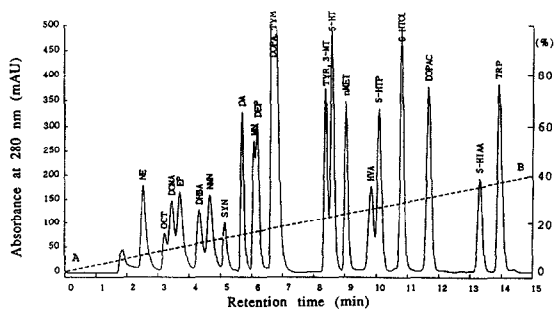


Fig. 2. HPLC pattern with UV detection for the standard compounds listed in Table I. Column specifications and composition of mobile phase are given in the Experimental section. A 5-nmol aliquot of each compound was injected.

RESULTS AND DISCUSSION

After HPLC separation of the standard compounds, initial detection was accomplished by UV

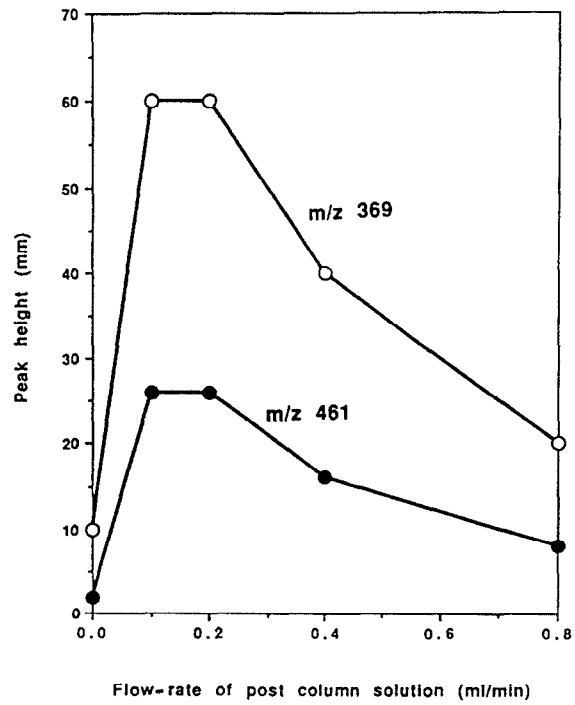


Fig. 3. Effect of post-column addition on the intensity of matrix ions in HPLC–CF-FAB-MS. Composition of mobile phases is given in the Experimental section. Solution added post-column consisted of 1% glycerol in methanol (v/v).

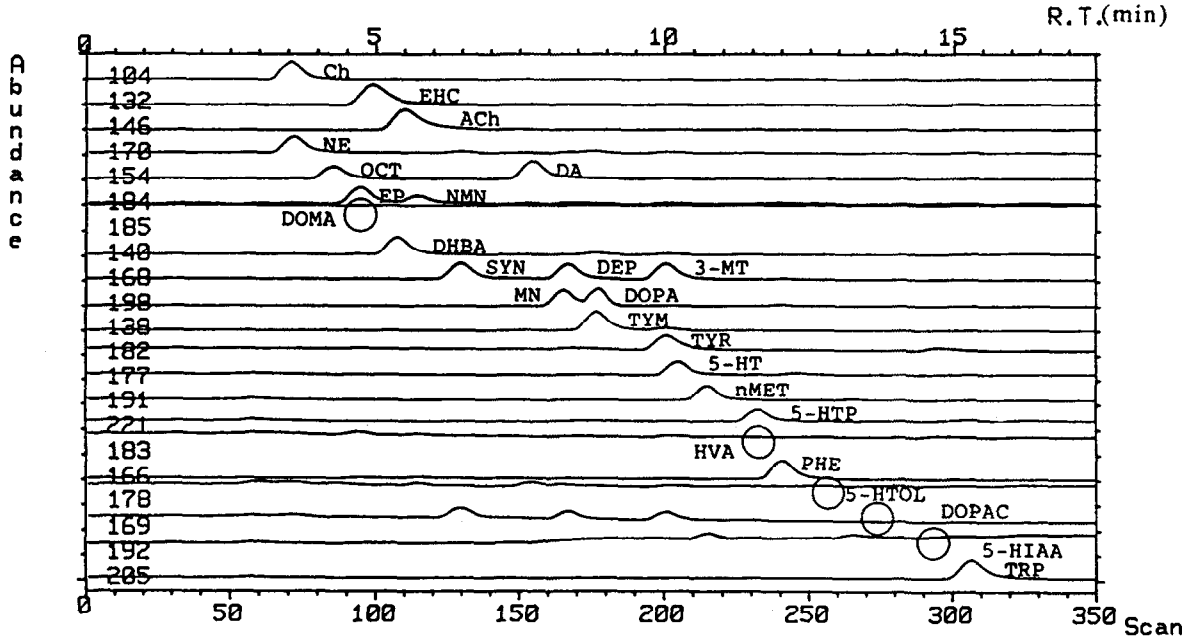


Fig. 4. Mass chromatograms for HPLC–CF-FAB-MS analysis of the standard compounds listed in Table I. Column specifications and composition of mobile phase are given in the Experimental section. A 5-nmol aliquot of each compound was injected. Positions on the traces marked with a circle represent the retention time for compounds not detected for the sample size used in this analysis.

since most of the neurochemicals of interest to us absorb near 280 nm. We found that, when only solvent A was utilized, retention times were extremely long, and broad peaks were observed for the strongly retained compounds. Gradient addition of methanol containing 0.2% TFA along with 1% glycerol led to faster elution of these substances. After examining various mixing rates for the two solvent systems, we ascertained that the linear gradient system of 0–40% solvent system B gave optimal separation in an acceptable amount of time. A typical HPLC profile using UV detection is shown in Fig. 2. Peaks for Ch, ACh, EHC and PHE were not observed at 280 nm because these compounds lack a chromophore at that wavelength; consequently, MS was used for detection of these substances. With the solvent system described above, HPLC separation of the analytes was completed within 15 min. As can

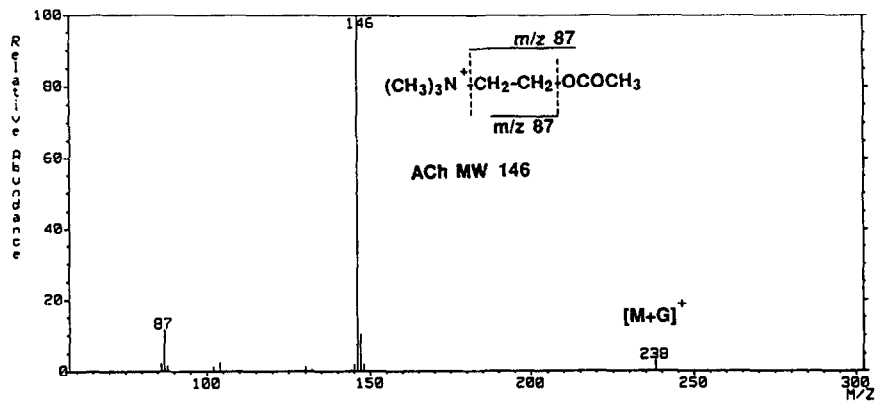


Fig. 5. CF-FAB mass spectrum of acetylcholine (ACh); G = glycerol.

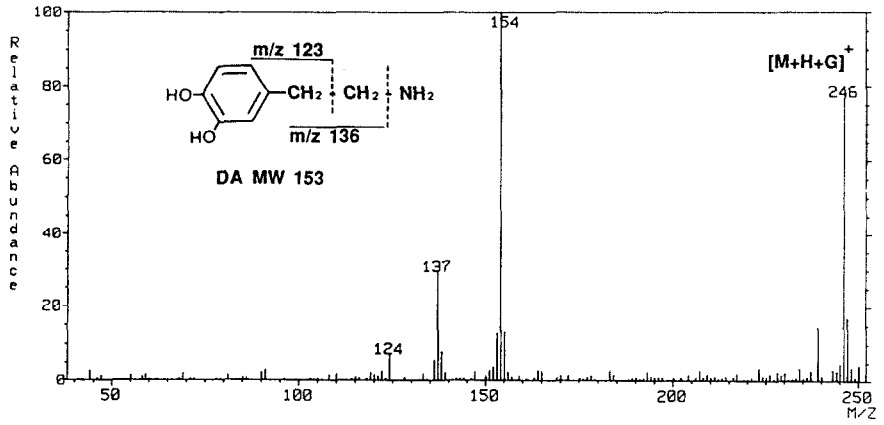


Fig. 6. CF-FAB mass spectrum of dopamine (DA); G = glycerol.

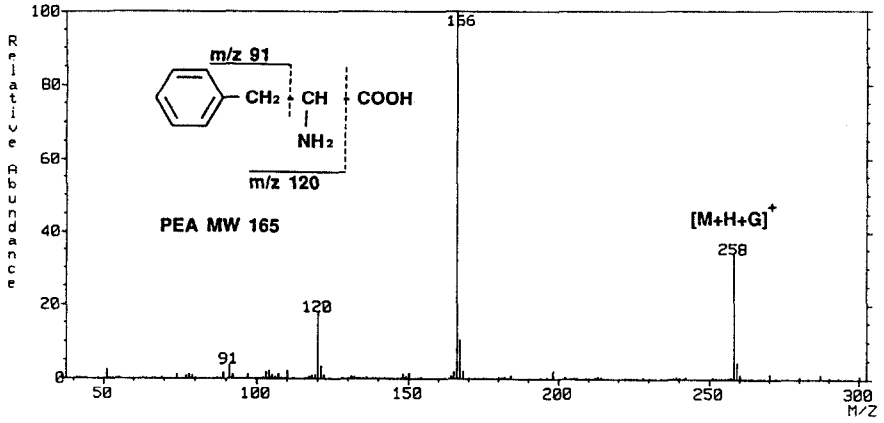


Fig. 7. CF-FAB mass spectrum of phenylalanine (PHE); G = glycerol.

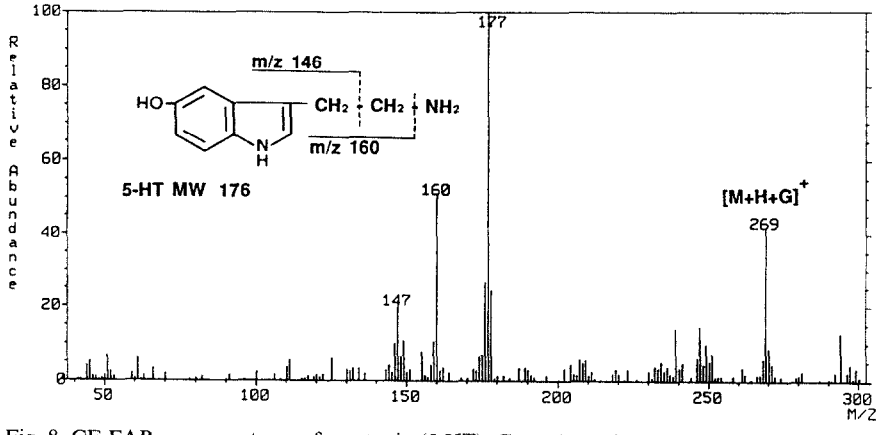


Fig. 8. CF-FAB mass spectrum of serotonin (5-HT); G = glycerol.

TABLE II

RELATIVE RESPONSE OF SELECTED NEUROCHEMICALS TO DETECTION BY FRIT-FAB-MS

Compound[a]	Monitored ion (*m/z*)	Peak area[b]	Response[b,c] (%)
ACH	146	42832	100.0
EHC	132	39187	91.5
PEA	122	25123	58.7
Ch	104	19184	44.8
PHE	166	14675	34.3
PEOHA	138	13523	31.6
TM	161	6046	14.1
3-MT	168	5449	12.7
TRP	205	4427	10.3
DEP	168	4216	9.8
SYN	168	4019	9.4
TYM	138	3838	9.0
MN	198	3762	8.8
EP	184	2889	6.7
DA	154	2386	5.6
TYR	182	2334	5.4
nMET	191	2033	4.7
OCT	154	1520	3.5
NMN	184	1361	3.2
DOPA	198	1337	3.1
NE	170	1313	3.1
5-HT	177	1033	2.4
DHBA	140	920	2.1
5-HTP	221	762	1.8
HVA	183	390	0.9
5-HIAA	192	239	0.6
5-HTOL	178	214	0.5
DOPAC	169	55	0.1
DOMA	185	n.d.	n.d.

[a] Abbreviations as shown in Table I.
[b] n.d. = Not detected.
[c] Response relative to ACh.

be seen from the chromatogram in Fig. 2, the close similarities in chemical structures of many of the compounds makes it difficult to obtain complete HPLC separation, as evidenced by closely eluting or overlapped peaks. However, the use of CF-FAB-MS for detection eliminates this problem. By using SIM, differentiation between these compounds can be readily accomplished even without baseline HPLC separation. Moreover, even for isobaric sets of compounds such as OCT and DA (mol wt. 153), EP and NMN (mol. wt. 183), SYN, DEP and 3-MT (mol. wt. 167) and MN and DOPA (mol. wt. 197) the HPLC retention times are sufficiently separated that the specificity afforded by MS permits facile peak identification.

In order to increase efficiency and ion stability, it was necessary to employ post-column addition of a mixture of 1% glycerol in methanol. The effect of flow-rate of the post-column solution on the production of matrix ions by CF-FAB was examined by monitoring the intensities of ions at *m/z* 369 (protonated glycerol tetramer) and *m/z* 461 (protonated glycerol pentamer). As shown in Fig. 3, the maximum peak heights for both of these ions were obtained when the flow-rate was 0.1–0.2 ml/min.

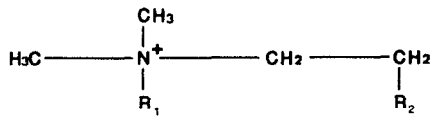
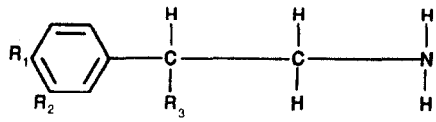
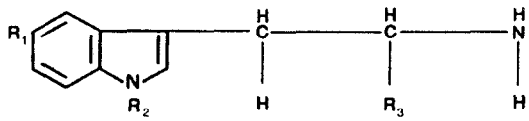
The mass chromatograms obtained for HPLC–CF-FAB-MS analysis of the selected neurochemicals are presented in Fig. 4. For the quaternary ammonium compounds Ch, ACh and EHC molecular cations ($[M]^+$) at *m/z* 104, 146 and 132, respectively, were monitored. For the other neurochemicals, protonated molecular ions ($[MH]^+$) were detected. The retention times of the compounds being tested, as recorded by UV and CF-FAB-MS are listed in Table I. As can be seen in the table, longer retention times were observed for the peaks detected by MS (Fig. 4) as compared with UV (Fig. 2) because of the additional length of tubing in between the UV detector and the CF-FAB probe.

CF-FAB mass spectra of representative compounds are shown as follows: ACh (quaternary amine), Fig. 5; DA (catecholamine), Fig. 6; PHE (amino acid), Fig. 7; and 5-HT (indoleamine), Fig. 8. Proposed cleavages for each analyte are illustrated for each structure; however, details of the fragmentation mechanisms have not yet been comprehensively investigated. From the spectra it can be seen that the base peak was either $[M]^+$ or $[MH]^+$, depending on the compound class.

In order to ascertain the sensitivity of CF-FAB-MS for our compounds of interest, 5 nmol of each substance were injected into the HPLC system, and the retrieved area for each base peak was measured after SIM. The results are summarized in Table II. The data show that the highest sensitivity was obtained for quaternary ammonium compounds such as ACh and EHC. The detection limit for ACh was less than 1 pmol. The poorest sensitivities were found for compounds such as HVA, 5-HIAA, 5-HTOL, DOPAC and DOMA, all of which lack an amino group. These compounds were either not observed or were only marginally detected at the 5-

TABLE III

RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND RESPONSE FOR ANALYSIS BY CF-FAB-MS

Compound	R_1	R_2	R_3		Peak area	Response (%)
Quaternary ammonium compounds						
AcH	CH_3	$OCOCH_3$			42 832	100.0
EHC	C_2H_5	CH_2OH			39 187	91.5
Ch	CH_3	OH			19 183	44.8
Catechol analogues						
PEA	H	H	H		25 123	100.0
PEOHA	H	H	OH		13 523	53.8
TYM	OH	H	H		3838	15.3
DA	OH	OH	H		2386	9.5
NE	OH	OH	OH		1313	5.2
Indole analogues						
TM	H	H	H		6046	100.0
TRP	H	H	COOH		4427	73.2
nMET	OH	CH_3	H		2033	33.6
5-HT	OH	H	H		1033	17.1
5-HTP	OH	H	COOH		764	13.6

nmol level. We found approximately a 1000-fold difference in sensitivity between ACh and DOPAC.

From a review of our data, we ascertained that the sensitivity of individual compounds using our CF-FAB-MS method could be correlated with chemical structure. For example, the response for PHE [C_6H_5–CH_2–$CH(NH_2)$–COOH] was almost ten times greater than for DOPA [$C_6H_3(OH)_2$–CH_2–$CH(NH_2)$–COOH], with the structures of these related compounds differing only by the presence of two hydroxyl groups on the phenyl side-chain of DOPA. In the current study, it was apparent that compounds with alcoholic or phenolic substituents tended to exhibit lower responsiveness by CF-FAB-MS; in general, the greater the hydrophobic nature of a compound, the stronger the signal CF-FAB-MS. This trend is substantiated by the data presented in Table III for representative quaternary ammonium, catechol and indole analogues.

In conclusion, we present methodology for LC separation and CF-FAB-MS quantitation of multiple neurochemically important compounds. We believe that this technique will be quite valuable for monitoring changes in the central nervous system during behavioral, pharmacological and psychological investigations if the sensitivity of the technique can be improved. In future studies we plan to explore the use of packed capillary columns for HPLC separation in order to eliminate the need for post-column splitting. In addition, we will examine the use of negative-ion detection in conjunction with CF-FAB-MS analysis of these compounds in an effort to enhance the sensitivity for the more hydrophilic analytes and to obtain more uniform responsiveness for the different structural analogues.

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REFERENCES

1 C. R. Blakley and M. L. Vestal, *Anal. Chem.*, 55 (1983) 750.
2 R. D. Voyksner and C. A. Haney, *Anal. Chem.*, 57 (1985) 991.

3 R. P. W. Scott, C. G. Scott, M. Munroe and J. Hess, Jr., *J. Chromatogr.*, 99 (1974) 395.
4 M. J. Hayes, E. P. Lankmayer, P. Vouros, B. L. Karger and J. M. McGuire, *Anal. Chem.*, 55 (1983) 1745.
5 D. E. Games, *Biomed. Mass Spectrom.*, 8 (1981) 454.
6 T. Mizuno, T. Kobayashi, Y. Ito and D. Ishii, *Mass Spectros.*, 35 (1987) 9.
7 Y. Ito, T. Takeuchi and D. Ishii, *J. Chromatogr.*, 358 (1986) 201.
8 Y. Ikarashi, K. Itoh and Y. Maruyama, *Biomed. Environ. Mass Spectrom.*, 20 (1991) 21.