ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 729–738

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Single pump column switching technique employing a flow gradient and wavelength programmed fluorescence for simultaneous monitoring of serotonin, fluoxetine and norfluoxetine in rat brain microdialysate

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Received 10 January 1996; accepted 12 July 1996

Abstract

A single pump column switching technique with multidimensional chromatography, flow gradient and wavelength programmed fluorescence detection was developed for simultaneous quantitation of serotonin, fluoxetine and norfluoxetine in rat brain microdialysate. The column switching was configured such that position I of the switching valve employed column I (50 mm length) and column II (250 mm length) in series. This configuration resulted in optimal resolution of serotonin from interfering neurochemicals from rat brain. After elution of serotonin at 13.2 min the valve was switched to position II in which the flow of the mobile phase was directed through column I only. Flow gradient programming was then used to ramp the flow rate from 0.1 to 0.4 ml min^{-1} which resulted in optimal elution of fluoxetine and norfluoxetine. Strategic optimization of the single mobile phase enabled use of a single pump and detector making the analytical system simple and cost effective. Wavelength programmed fluorescence enabled sensitive detection of the analytes despite the difference in their fluorescence spectrum. The limit of detection for serotonin, norfluoxetine and fluoxetine were 10, 612 and 523 fmol, respectively. Rat brain microdialysate samples demonstrated selectivity for serotonin, fluoxetine and norfluoxetine. The method demonstrates application to the study of site specific neuropharmacokinetics and neuropharmacodynamics of fluoxetine in vivo. © 1997 Elsevier Science B.V.

Keywords: Reversed phase chromatography; Single pump column switching; Flow gradient; Ion pair; Wavelength programmed fluorescence; Fluoxetine; Serotonin; Rat brain microdialysate

1. Introduction

Microdialysis sampling is a powerful technique for the study of the neurochemical effects of drugs in vivo [1]. High performance liquid chromatogra-

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PII S0731-7085(96)01898-5

phy and the microdialysis sampling approach are complementary laboratory tools. Microdialysis provides a highly filtered protein free, low volume solution of analytes which can be directly injected into the chromatographic system. Fluoxetine (Prozac) is a selective serotonin reuptake inhibitor widely used in antidepressant therapy and obsessive compulsive disorder [2]. Fluoxetine causes selective inhibition of the uptake carrier on serotonin nerve terminals and this results in an increased concentration of serotonin in the extracellular fluid sampled by microdialysis [3]. This dynamic response indicated by changes in the level of serotonin in physiological active systems in vivo is critical to the study of drug effects in neuropharmacology. The time course of drug concentration in the brain (neuropharmacokinetics) has a profound influence on the serotonin concentration in the rat brain studied by microdialysis. Quantitation of serotonin, fluoxetine and norfluoxetine (pharmacologically active metabolite) simultaneously would allow the study of the time course of drug action on serotonin levels in a physiologically dynamic system in vivo.

There are no analytical methods available in the literature for simultaneous detection of serotonin, fluoxetine and norfluoxetine. Quantitative determination of serotonin, fluoxetine and norfluoxetine simultaneously in rat brain microdialysate presents a significant bioanalytical challenge because a relatively low concentration of serotonin must be determined in a matrix which has a high concentration of other potentially interfering neurochemicals. Also, fluoxetine and norfluoxetine are lipophilic in nature and have different chromatographic and fluorescent spectral properties as compared to serotonin. The structure of the analytes can be observed in Fig. 1. The isocratic separation of such samples may exhibit poor resolution of early eluting peaks, with difficult detection of late-eluting bands and unnecessarily long separation times. A solution to this problem would be to change conditions during the separation to allow independent optimization of the k' values for the different chromatographic peaks. This can be approached by using either gradient elution or column switching techniques [4].

Several analytical methods exist for quantitation of fluoxetine and norfluoxetine in human plasma. Lantz et al. [5], Nash et al. [6] and Lopez et al. [7] have developed gas chromatographic methods with electron capture detection. Suckow et al. [8] developed a precolumn fluorescent derivatization method with dansyl chloride and Orsulak et al. [9] developed an HPLC method with ultraviolet absorption detection for quantitation of fluoxetine and norfluoxetine in human plasma. Fluoxetine is administered as a racemate and the enantiomers of fluoxetine are similarly effective in the in vivo and in vitro inhibition of serotonin uptake in rats [10]. Potts et al. developed a sensitive method for the quantitation of the enantiomers of fluoxetine and norfluoxetine in human and animal tissue [11]. The above methods are unsuitable for use with microdialysis because of either the sample pretreatment steps that are necessary or a lack of sensitivity. Quantitative methods for determination of serotonin include gas chromatography with mass spectroscopy [12], radioimmunoassay [13] and HPLC with fluorescence [14] and electrochemical detection [15]. Electrochemical detection though sensitive for serotonin suffers from a lack of ruggedness and is incompatible with flow gradient and column switching which cause disequilibrium of the electrochemical flow cell with the mobile phase.

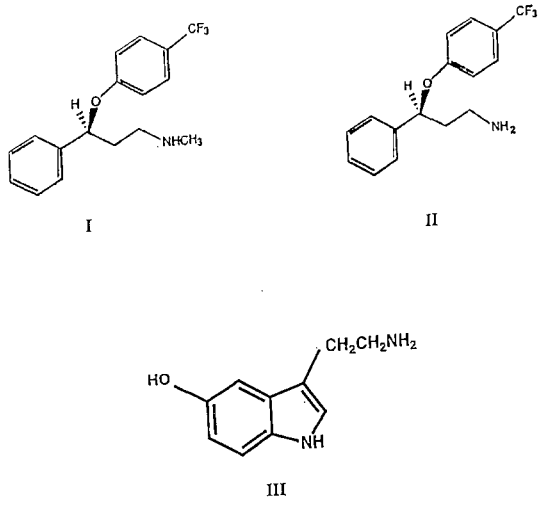

Fig. 1. Chemical structures of (I) fluoxetine, (II) norfluoxetine and (III) serotonin.

This paper reports the development and application of a novel single pump column switching technique for simultaneous monitoring of serotonin, fluoxetine and norfluoxetine in rat brain microdialysate. A flow gradient was used to enable early elution of relatively lipophilic analytes. The native fluorescent properties of the analytes were exploited to allow detection of low concentrations in rat brain microdialysate. Enhancement of sensitivity was further achieved by use of microbore chromatography and optimization of the flow cell volume. The use of the wavelength program enabled simultaneous detection of the analytes with optimum sensitivity. This is the first description of the use of a single pump column switching technique for analysis of compounds possessing large differences in retention times. This approach offers an alternative to conventional column switching which requires the use of two pumps and two switching valves. The successful implementation of this approach requires optimization of the mobile phase such that a single mobile phase is suitable for both chromatographic systems.

2. Materials and methods

Serotonin was purchased from Sigma (St. Louis, MO, USA). Fluoxetine and norfluoxetine were supplied by Eli Lilly and Company. Glacial acetic acid was purchased from J.T. Baker Chemical (Phillipsburg, NJ, USA). Acetonitrile, tetrahydrofuran and methanol were purchased from Baxter Healthcare (McGraw Park, IL, USA). The ion pairing agents heptane sulfonic acid sodium salt, 1-octane sulfonic acid, sodium salt, decyl sodium sulphate and sodium dodecyl sulphate were purchased from Eastman Kodak (Rochester, NY, USA). All reagents were of HPLC grade unless stated otherwise.

2.1. Spectral characterization

The spectral properties of serotonin, fluoxetine and norfluoxetine were determined using a Perkin Elmer model LS-50 scanning luminescence spectrometer equipped with a xenon excitation source (Perkin Elmer Corporation, Rockville, MD, USA). The excitation maxima for the analytes in the mobile phase were determined by scanning from 200 to 500 nm with the emission monochromator set to zero. The emission spectrum was scanned after setting the excitation monochromator to the maximum wavelength.

2.2. HPLC system

The chromatographic system consisted of a Hewlett Packard model 1050 pump (Hewlett-Packard, Avondale, PA, USA), Autochrome model 401 six port switching valve (Autochrom, Milford, MA, USA) equipped with a model 201 solenoid interface and a Rheodyne model 7161 injector equipped with a 5 μl sample loop. Column I was a Nucleosil C_{18} (2.0 × 50 mm, 3 μm, Phenomenex, Torrence, CA, USA). Column II was a Supelcosil C_{18} (2.1 × 250 mm, 5μm, Supelco, PA, USA). Chromatographic data was acquired on a Hewlett packard integrator model 3396 A. The valve switching, flow gradient and wavelength programmed fluorescence were controlled by contact closures on the injector.

2.3. Mobile phase

The optimal mobile phase used for separation consisted of 0.33 mM 1-octane sulfonic acid sodium salt in acetic acid (65 mM, adjusted to pH 2.8 with glacial acetic acid) and acetonitrile (67:33, v/v). The mobile phase was filtered and degassed by helium sparging.

2.4. Wavelength programmed fluorescence detection

Fluorescence detection with the excitation wavelength at 280 nm and emission wavelength at 340 nm was used for serotonin. After elution of the serotonin peak, the excitation wavelength was changed by detector program to 235 nm and the emission wavelength to 280 nm. The excitation bandwidth was 18 nm and the emission bandwidth was 40 nm. The detector gain was set at 100 and the time constant at 2 s.

2.5. Flow gradient programming

The mobile phase flow rate was maintained at 0.1 ml min^{-1} with the switching valve at position I. After elution of the serotonin peak the flow of the mobile phase was ramped from 0.1 ml min^{-1} to 0.4 ml min^{-1} by programming a flow gradient with the HP model 1050 pump.

2.6. Column switching operation

A schematic representation of the single pump column switching technique is shown in Fig. 3. The switching valve was pneumatically activated and its position controlled by use of a system controller, interfaced to the switching valve by a solenoid interface. The switching valve positions used during the analysis are shown in Fig. 3. The configuration of the switching valve was designed such that in position 1, column I and column II were in series. After elution of the serotonin peak at 13.2 min the valve was switched to position 2, which was designed such that the flow of mobile phase proceeded through column I only.

3. Rat brain surgery and microdialysis sample collection

Male Sprague Dawley Rats (250–350 g) were housed under diurnal lighting. Animals were given ad libitum access to food and water. Rats pretreated with atropine (1 mg kg^{-1}, intraperitoneally) were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, intraperitoneally). The head was shaved and placed in a stereotaxic device. A small hole was drilled through the skull and an intracerebral guide cannulae (CMA/12, BAS, West Lafayette, IN, USA) was inserted into the anterior lateral striatum. The spatial coordinates were 2 mm anterior to the bregma, 3 mm from the mid-sagittal suture and 6 mm ventral from the dura [16]. The length of the probe (3 mm) was taken into account while fixing the coordinates and the coordinates of implantation were at the tip of the guide cannula. After implantation, the cannula was fixed firmly to the skull with anchor screws and dental cement and then occluded with a obdurator. The dialysis experiments were performed at least 3 days after the surgery to allow the animals to recover. On the day of the experiment the obdurator was removed and the microdialysis probe (CMA/12 probe, 3 mm length, BAS, West Lafayette, IN, USA) was inserted into the striatum via the guide cannulae. The dialysis membrane consisted of a polycarbonate/polyether copolymer. The inner diameter of the membrane was 400 μm and the molecular weight cutoff was 20 000. The probe was perfused continuously with perfusion fluid (Na^+ 147 mM, K^+ 3.5 mM, Ca^{2+} 1.0 mM, Mg^{2+} 1.2 mM, Cl^- 129 mM, phosphate 1 mM, HCO^- 25 mM) via polyvinyl tubing attached to the implant on the rat's head with a microperfusion pump (CMA 100, Carnegie Medicin, Sweden) at a flow rate of 1 μl min^{-1}. The animals were placed in a plexiglass container during the experiment and allowed free access to food and water. A 4-h period was allowed for baseline stabilization following the probe insertion. Fractions of the dialysis solution were collected over a period of 30 min with an automated fraction collector and analyzed immediately following collection. The dialysate was collected for a period of 8 h following implantation of the microdialysis probe.

4. Results and discussion

4.1. Enhancement of detectability

The limit of detection (LOD) obtained with a conventional column (4.6 mm internal diameter) was compared with that obtained with a minibore column (2.1 mm internal diameter). When serotonin was used as a model analyte the LOD obtained with a conventional column was 189 pg whereas the LOD obtained with minibore column was 58 pg. The use of a minibore column resulted in a 3.25-fold increase in mass sensitivity for serotonin. For this experiment the McPherson Fluorescence detector (model FL-750) was used. Replacement of the 5 μl flow cell with a 16 μl flow cell resulted in a 3-fold increase in the signal to noise ratio. Thus minibore chromatography along with flow cell volume optimization resulted in a 9.75-fold increase in sensitivity.

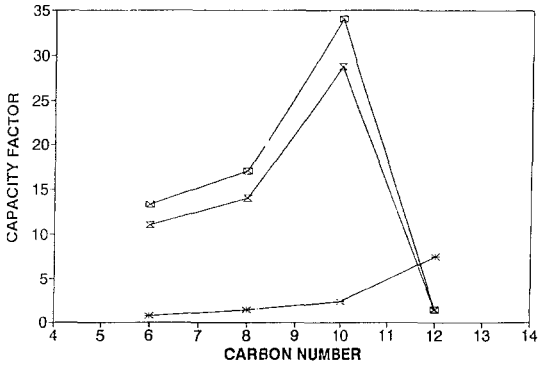

Fig. 2. Effect of carbon number of ion pairing agent on capacity factor. (*, Serotonin; □, Norfluoxetine; ◆, Fluoxetine).

5. Separation optimization

Serotonin, fluoxetine and norfluoxetine (Fig. 1) possess ionizable amine functional groups which can be used to enhance their retention selectivity by addition of an oppositely charged anionic pairing ion to the mobile phase. Optimization of separation conditions for simultaneous quantitation of serotonin, fluoxetine and norfluoxetine was achieved by investigating the effects of size of the ion pairing agent, the concentration of the ion pairing agent, the concentration and types of organic modifiers, flow gradient programming, mobile phase gradient and the single pump column switching technique developed in our laboratory.

5.1. Effect of ion pairing agent

The retention of the three analytes was investigated by studying the effect of the pairing ion hydrophobicity (carbon number) at a constant concentration of 0.33 mM in a mobile phase consisting of acetic acid (65 mM, adjusted to pH 2.8 with glacial acetic acid) and acetonitrile (67:33, v/v). The anionic pairing agents studied were heptane sulfonic acid, sodium salt (C_6), 1-octane sulfonic acid, sodium salt (C_8), decyl sodium sulfate (C_{10}) and sodium dodecyl sulphate (C_{12}). Fig. 2 represents the effect of increasing the carbon number of the ion pairing agent on the capacity factor. An increase in the retention times of serotonin, norfluoxetine, and fluoxetine was observed when the length of the ion pairing agent was increased from C_6 to C_{10}. When C_{12} was used as an ion pairing agent the capacity factor of fluoxetine and norfluoxetine decreased. A possible explanation for the decrease in the capacity factor of fluoxetine and norfluoxetine could be due to the formation of micelles. In reversed phase chromatography, retention is related to the magnitude of substrate partitioning into the stationary phase with hydrophobic molecules retained to a greater extent. Two additional partition process are possible when micelles are added to the mobile phase. The partition pathways become (1) partition between the aqueous and the stationary phase (2) partition between the aqueous and the micellar phases and (3) partition between the micellar phase and the stationary phase [17]. Fluoxetine and norfluoxetine possess an electron withdrawing trifluoromethyl (-CF_3) functional group on the benzene ring which could cause polarization of the molecule and subsequent interaction with the micelle by coulombic attraction. Further optimization experiments were carried out with C_8 as an ion pairing agent due to the reasonable analysis time of less then 18 min. The goal of the optimization experiments was to reduce the elution window between serotonin ($k' = 1.3$), norfluoxetine ($k' = 14$) and fluoxetine ($k' = 17$). The dependence of capacity factor (k') on the concentration of the counter-ion was studied in the concentration range from 0.15 to 0.77 mM. No concentration dependent effect of the counter-ion concentration could be observed. The concentration dependency of k' on ion pairing agent concentration would probably have been observed if the concentration range had been greater. Observation from experimental data suggests that at concentrations above 0.15 mM of 1-octane sulfonic acid, sodium salt the capacity factors for serotonin, norfluoxetine and fluoxetine were saturated at values of 1.3, 14 and 17, respectively. A lower concentration of the ion pairing agent would have decreased the capacity factors for all the analytes and the k' for serotonin would be less then 1.3. This would cause elution of serotonin at the solvent front and because of this, a greater concentration range was not studied.

5.2. Effect of the organic modifier on k'

In order to reduce the elution window between serotonin and norfluoxetine an organic modifier was required which would selectively reduce the retention of fluoxetine and norfluoxetine without effecting serotonin. Tetrahydrofuran, a relatively nonpolar solvent, was evaluated. Fluoxetine and norfluoxetine are strongly hydrophobic relative to serotonin, theoretically a non polar solvent like tetrahydrofuran would decrease the retention of fluoxetine and norfluoxetine relative to that of serotonin. The effect of tetrahydrofuran was studied with a ternary mobile phase consisting 0.33 mM 1-octane sulfonic acid, sodium salt in acetic acid (65 mM, pH 2.8 adjusted with glacial acetic acid) tetrahydrofuran and acetonitrile (5:33:62 v/v/v). At a concentration of 5% tetrahydrofuran the capacity factor of serotonin, fluoxetine and norfluoxetine were 0.9, 6.9 and 7.5, respectively with inadequate resolution between fluoxetine and norfluoxetine. At a concentration of 2.5% tetrahydrofuran the capacity factors for the above analytes were 1.0 for serotonin, 9.3 for norfluoxetine and 10.5 for fluoxetine but the resolution between the fluoxetine and norfluoxetine peaks was not adequate. Inclusion of tetrahydrofuran in the mobile phase reduced the elution window between serotonin and norfluoxetine but this was only achieved at the expense of poor resolution between norfluoxetine and fluoxetine.

5.3. Evaluation of gradient elution

Gradient elution is often used when complex samples are encountered that have a wide range of retention behavior. The mobile phase was maintained at 25% acetonitrile: 75% distilled deionized water containing 65 mM glacial acetic acid (pH 2.8) and 0.33 mM 1-octane sulfonic acid, sodium salt for the first 2 min and 50% acetonitrile: 50% distilled deionized water containing 65 mM glacial acetic acid and 0.33 mM 1-octane sulfonic acid, sodium salt from 2 to 28 min. The gradient resulted in baseline drift and retention times of 23.5 and 26.3 min for norfluoxetine and fluoxetine, respectively. Another significant problem encountered with the use of the gradient was that a reequilibration time of 30 min was required between successive injections yielding total analysis time of 56.3 min per sample. The use of a mobile phase gradient therefore did not solve the separation problem efficiently and was considered to be a less than desirable option.

5.4. Flow gradient

A time programmed flow gradient was set up with the Hewlett Packard 1050 pump so that the flow rate was initially set at 0.2 ml min^{-1} and after elution of the serotonin peak the flow rate was increased to 0.3 ml min^{-1}. The chromatographic conditions employed were a Nucleosil C_{18} column (Phenomenex, 2.0 mm I.D × 50 mm length) with a mobile phase containing 0.33 mM octane sulfonic acid, sodium salt in acetic acid (65 mM, pH 2.8 with glacial acetic acid) and acetonitrile (67:33, v/v). The analysis time under these chromatographic conditions was 12 min. The method was considered to be optimal for standards, but when rat brain microdialysate samples were injected, serotonin was unresolved from early eluting neurochemicals originating from the matrix. The blank rat brain microdialysate showed an absence of interfering peaks at the retention times of fluoxetine and norfluoxetine. The chromatographic system mentioned above with column I is therefore optimal for quantitation of fluoxetine and norfluoxetine but inadequate to quantitate serotonin because of interference from endogenous neurochemicals.

6. Optimization of chromatographic conditions for system II

Optimization of the conditions for resolving serotonin from interfering neurochemicals was achieved by investigation of the effects of the pH of the mobile phase and the concentration of the ion pairing agent. The optimization was performed on a Supelcosil LC-18 DB column (2.1 mm I.D × 250 mm). The dependence of capacity factor on pH of the mobile phase was studied with a mobile phase containing 0.33 mM octane sulfonic acid, sodium salt in acetic acid (65 mM,

pH 2.8 adjusted with glacial acetic acid) and acetonitrile (67:33 v/v). In the pH range studied from 3 to 7 no effect of pH on capacity factor was observed. This could likely be attributed to the complete ionization of the analytes in the pH range studied. The effect of the concentration of ion pairing agent was studied from 0.15 to 0.77 mM and no concentration dependent effect of the ion pairing agent could be observed. The retention time for serotonin on this system was 7 min and serotonin could be resolved from potentially interfering neurochemicals on chromatographic system II. The above chromatographic system with column II was inappropriate for simultaneous analysis however, because of the unreasonably long analysis time for fluoxetine and norfluoxetine of 68 min.

6.1. Single pump column switching technique

Column I (Nucleosil C_{18} 2.0 mm I.D × 50 mm length) was optimal for separation of fluoxetine and norfluoxetine in terms of the analysis time but inappropriate for resolving serotonin from interfering neurochemicals. Column II (Supelcosil LC-18 DB, 2.1 mm I.D × 250 mm) was optimal for resolving serotonin from interfering neurochemicals but inadequate for fluoxetine and norfluoxetine due to their long retention times on this system. The use of the short column (column I) resulted in a lower k' for the late eluting components and the use of the longer column (column II) resulted in increased resolution. Coupling these two columns via a column switching approach provides a strategy for the optimization of the separation. The classical approach would be to use conventional heart cut column switching which would require the use of two pumps and two valves. In order to simplify the system, a unique design using a single pump, single valve and a single detector was developed. Fig. 3 represents a schematic diagram of the single pump HPLC with column switching. The design consists of an Autochrome model 401 six port switching valve. Column I was connected to position 6 of the switching valve and position 4 and 5 were connected by tubing (0.007 inch I.D.). Column II was connected between position 1 and 2 of the six port switching valve. Position 3 of the switching valve was connected to the fluorescence detector. The configuration was such that the flow of mobile phase was through column I and column II and finally to the detector in position I, which provides the high resolution necessary for separation of serotonin from interfering neurochemicals. After elution of serotonin the valve is switched to position II, in which the flow of mobile phase is through column I, then the loop of the switching valve and finally to the detector. This configuration enabled earlier elution of fluoxetine and norfluoxetine with flow gradient programming. In position II there is no flow of mobile phase through column II. It was first thought that this might cause disequilibrium of the column with the mobile phase and result in irreproducible retention times. The reproducibility of the retention times for the various analytes was investigated. The relative standard deviations for the retention times of the analytes were less then 3% when a standard solution of the analytes was injected ($N = 6$), demonstrating the reproducibility of the retention times. The procedure was implemented by strategic optimization of the mobile phase such that a single mobile phase was suitable for both chromatographic systems. This is the primary condition necessary for the system to work. Optimization experiments carried out with the two columns revealed that k' was independent of the concentration of the ion pairing agent in the concentration range studied from 0.15 to 0.77 mM. In addition, retention of the analytes was not affected in the pH range studied from 2.5 to 7. This phenomenon was exploited to develop a single mobile phase optimal for simultaneous quantitation of serotonin, fluoxetine and

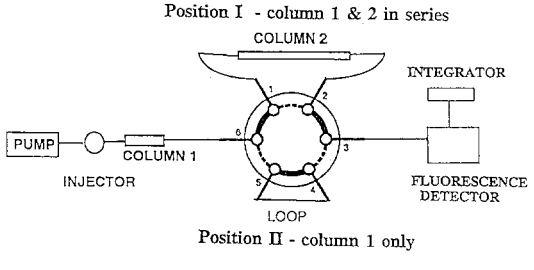

Fig. 3. Design of single pump column switching technique.

norfluoxetine. The optimal mobile phase for the chromatographic systems consisted of 0.33 mM 1-octane sulfonic acid, sodium salt in acetic acid (65 mM, pH 2.8 adjusted with glacial acetic acid) and acetonitrile (67:33 v/v). This mobile phase was optimized for the chromatographic system I and system II individually so as to obtain resolution of serotonin from interfering neurochemicals and to enable faster elution of norfluoxetine and fluoxetine. Increasing the concentration of acetonitrile in the mobile phase from 33 to 40% reduced the analysis time to 16 min but the increased concentration of acetonitrile resulted in loss of resolution of serotonin from interfering neurochemicals. When the concentration of acetonitrile was reduced to 25% the analysis time of serotonin was increased to 20 min and the total analysis time was raised to 30 min. The final concentration of 33% acetonitrile was chosen so as to achieve reduced analysis times of 18.5 min for norfluoxetine and 19.7 min for fluoxetine without compromising on the resolution of serotonin.

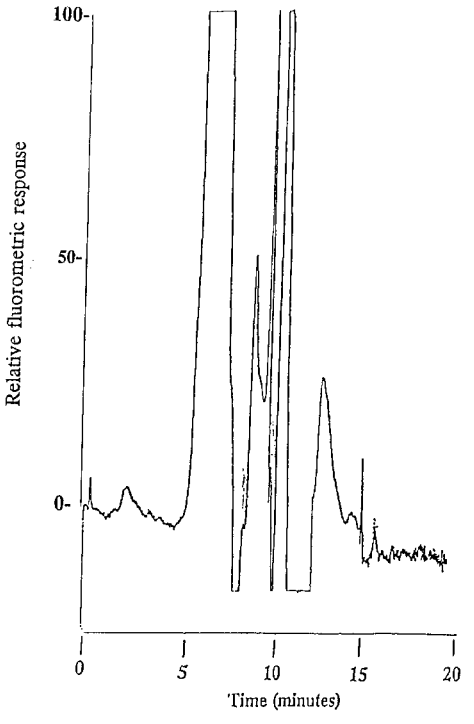

Fig. 4. Chromatogram of blank microdialysate obtained from anterior lateral striatum of rat brain.

6.2. Selectivity and detectability

The method was evaluated for selectivity by analysis of microdialysate samples obtained from the rat brain. The volume of the rat brain dialysate injected was 5 μl. The absence of interfering peaks during the elution windows for norfluoxetine at 18.4 min and fluoxetine at 19.6 min as can be observed from Fig. 4 and indicated selectivity for the analytes. Serotonin is an endogenous neurotransmitter and its presence is therefore observed in the blank. The limit of detection (LOD) represents the lowest concentration that can be determined to be statistically different from the analytical blank and was calculated at signal to noise ratio of 3 [18]. The limit of detection for serotonin, norfluoxetine and fluoxetine were 10, 612 and 523 fmol on column respectively. The limit of quantitation (LOQ) is the concentration above which quantitation can be carried out with adequate accuracy and precision. The estimate for LOQ was calculated using signal to noise ratio of 10. The LOQ obtained for the analytes were 33.3 fmol for serotonin, 2.03 pmol for norfluoxetine and 1.74 pmol for fluoxetine respectively. The peak height obtained for serotonin in Figs. 4 and 5 is approximately the same since these samples were collected 4 h after the microdialysis probe implantation and stabilization of serotonin baseline values was achieved.

6.3. Calibration

The matrix used to prepare standards in order to generate calibration curves consisted of blank microdialysate. The calibration curve for serotonin was found to be linear throughout the range of 0.020–0.525 pmol on column which was established as the range of interest. The mean correlation coefficient of the calibration curves ($n = 3$) was 0.998. A plot of log concentration versus log response demonstrated a slope of 1.02 which further validates linearity [19]. The calibration curve for norfluoxetine was found to be linear from 1.3 to 20 pmol. The mean correlation coefficient of the calibration curves ($n = 3$) was 0.997. A plot of

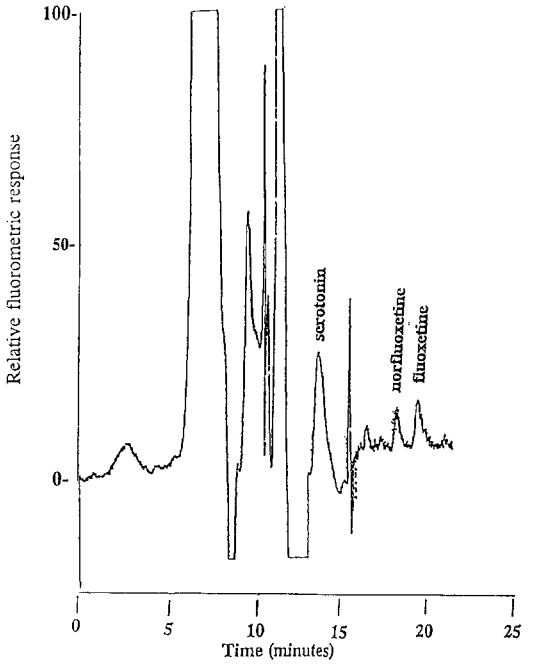

Fig. 5. Chromatogram demonstrating application of the single pump column switching technique for simultaneous monitoring of serotonin, norfluoxetine and fluoxetine. The values of serotonin, norfluoxetine and fluoxetine are 123 fmol, 2.66 and 2.62 pmol on column, respectively.

log concentration versus log response demonstrated a slope of 0.99. The calibration curve for fluoxetine was found to be linear from 1.2 to 20 pmol. The mean correlation coefficient of the calibration curves ($n = 3$) was 0.998. A plot of log concentration versus log response demonstrated a slope of 1.03.

6.4. Accuracy and precision

Accuracy of the method was evaluated by calculation of the percent difference of assayed values from spiked control concentrations (%DFA). Accuracy and precision of the method for serotonin, norfluoxetine and fluoxetine is shown in Table 1. The inter run precision of the method is within 11% and the accuracy is within 10% ($n = 3$) for all the three analytes. This demonstrates that the method is reproducible from run to run.

7. Conclusion

A novel HPLC method with single pump column switching coupled to flow gradient and wavelength programmed fluorescence detection was designed and developed for simultaneous quantitation of serotonin, fluoxetine and norfluoxetine in rat brain microdialysate. The method achieved selectivity for serotonin with regard to interfering neurochemicals in rat brain microdialysate with concurrent elution of fluoxetine and norfluoxetine in a reasonable analysis time. Fig. 5 represents a chromatogram of blank microdialysate obtained from anterior lateral striatum of rat brain spiked with norfluoxetine and fluoxetine and demonstrates application of the single pump column switching technique. The number of samples which can be analyzed using the method is 24 per day and the total run time for the simultaneous analysis of all three analytes

Table 1
Precision and accuracy of the method for serotonin, norfluoxetine and fluoxetine ($n = 6$)

Analyte	Spiked concentration (Pmol/5 μl)	Mean assayed concentration (Pmol/5 μl)	R.S.D. (%)	Accuracy (%)
Serotonin	0.031	0.029	10.3	8.5
	0.222	0.230	6.5	3.6
	0.508	0.489	4.7	3.7
Norfluoxetine	2.02	1.85	6.6	8.4
	7.58	7.3	4.8	3.6
	18.2	17.2	3.6	5.4
Fluoxetine	1.74	1.59	7.1	8.6
	6.51	6.18	5.2	5.0
	15.6	15.0	4.5	3.8

is 20 min. The method is sensitive enough for low level quantitation of serotonin in rat brain microdialysate and demonstrates potential for studying the neuropharmacokinetics and neuropharmacodynamics of fluoxetine in rat brain using microdialysis.

Acknowledgements

The authors would like to thank Dr James H. Johnson for his guidance with the microdialysis surgery and Eli-Lilly & Co. for the generous gift of fluoxetine and norfluoxetine.

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