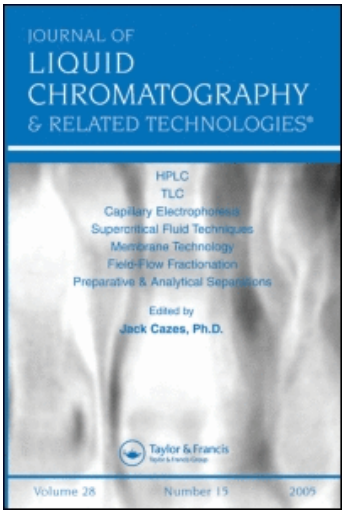

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THE ANALYSIS OF MELATONIN, TRYPTAMINE, AND SEROTONIN BY HPCE AND CEC

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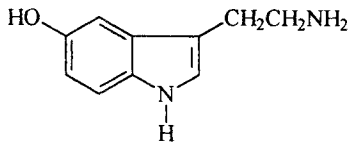
ABSTRACT

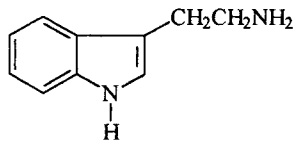
A capillary electrophoresis method has been developed for the analysis of melatonin, which depends on whether tryptamine and serotonin must be determined as well. If only a melatonin analysis is required, a bare capillary with a pH 4.41 buffer separates the analyte from the other two compounds, which are unresolved. If determination of all three species is necessary, a bare capillary with a pH 2.14 buffer is used. For better resolution of tryptamine and serotonin without the analysis of melatonin, a chemically modified capillary surface that lowers the electroosmotic flow is necessary.

INTRODUCTION

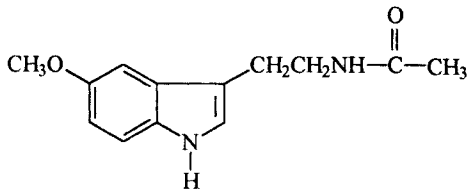
High performance capillary electrophoresis (HPCE) and capillary electrochromatography (CEC) are rapidly developing into valuable analytical techniques for the determination of a broad range of compounds. Continuous improvements in instrumentation, column technology, and detection are making the methods more reliable and versatile.



SEROTONIN



TRYPTAMINE



MELATONIN

Figure 1. Structures of analytes used in this study.

These developments are also leading to the evaluation of existing analytical protocols to determine if HPCE and/or CEC can provide better resolution or faster analysis time than is available from currently used methods, in particular, HPLC. For example, recently we reported on an HPCE method for the determination of aspartame in food, beverage and pharmaceutical products.[1] In comparison to the best reported HPLC protocol,[2] the HPCE analysis is compatible with the range of concentrations encountered in typical real world samples, can be done in less than one half the time, and suffers from no apparent interferences so that little or no sample preparation is necessary.

The determination of melatonin is another such practical analysis that might be improved by employing electroseparation systems. Melatonin is an important species in biological systems that has been investigated with respect to its properties and functions in the aging process and circadian rhythms.[3,4] Tryptamine and serotonin are often present with melatonin and have their own physiological significance. Therefore, a rapid and simple analytical method that can analyze for each of these species in the presence of the others is highly desirable. The structures for the three compounds are shown in Figure 1. The current chromatographic methods available for the analysis of melatonin include gas chromatography,[5] reverse phase HPLC with various detection options[6,7] and normal phase HPLC with fluorometric detection.[8] For the highest sensitivity,

radioimmunoassay has been the method of choice.[9] In the HPLC methods, the presence of tryptamine and serotonin can often be an interference in the analysis of melatonin because they frequently occur in the sample at concentrations 100 times higher than the analyte.[10]

Capillary electrophoretic separations in all modes have been described by Knox[11] and the analyst often has several choices available for a particular separation problem. For charged molecules, an unmodified fused silica capillary is often the simplest and most direct method of accomplishing a particular separation. In this format depending on the pH of the buffer, migration of the solute will be a combination of electroosmotic flow and electrophoretic mobility. However, some species, particularly bases, can be strongly adsorbed by the silanols on the column wall leading to poor recovery of the analyte and nonreproducible migration times. Another option is to use a coated capillary where an organic group is covalently bonded to the surface so that most of the silanols are either removed or shielded from the solute. A third option, particularly for some neutral compounds, is to use a packed capillary so that solutes are driven by electroosmosis but separated by interaction with a stationary phase on the particles (CEC).[12] A new form of CEC involves etching a capillary wall to increase the surface area and then bonding a stationary phase to this new surface.[13] This format is particularly suited to mixed-mode separations of charged molecules, i.e., a combination of differences in electrophoretic mobility and solute-bonded phase interactions. It has already been demonstrated that a wall bonded group, cyclodextrin, can be used for a CEC separation of enantiomers[14-18] in capillaries of similar diameter.

The goal of this study is two-fold. First, to determine if capillary electroseparation methods are suitable for the analysis of melatonin, tryptamine, and/or serotonin. Second, to compare, where possible, the different column formats in their separating ability for similar types of samples containing at least two of these analytes.

EXPERIMENTAL

Materials

Melatonin, serotonin, and tryptamine were purchased from Aldrich (Milwaukee, WI, USA) and used without further purification. A stock solution of 1 mg/mL of the mixture was prepared daily and working solutions for calibration curves as well as for testing of different capillaries were prepared by either dilution in the buffer or in water for sample stacking. Three buffers were used in the study: pH 2.14 consisted of 30 mM phosphate (phosphoric acid) and 19 mM Tris; pH 3.70 consisted of 30 mM citric acid and 25 mM β-alanine; pH

4.41 consisted of 30 mM acetic acid and 37.5 mM γ-amino-n-butyric acid. The working buffer was prepared by a 10-fold dilution of a stock solution. Water for the buffer was prepared on a Milli-Q apparatus (Millipore Corp., Milford, MA, USA). The buffer and samples were first sonicated and then degassed with helium.

The bare capillaries (50 μm, Polymico Technologies, Phoenix, AZ, USA and 75 μm, J & W Scientific, Folsom, CA, USA) were conditioned at the beginning of each day with a 5 min. rinse of 0.10 M NaOH followed by a 5 min. rinse with Milli-Q water and finally a 5 min. rinse with buffer. Three types of chemically modified capillaries were utilized: Poly[(N-acryloylamino) ethoxy]ethyl-β-D-glucopyranose (AEG),[19] octadecyl, and an etched diol.[13,20] For the chemically modified capillaries, the NaOH rinse was eliminated. Between runs the bare capillaries were rinsed for 2 min. with 0.10 M NaOH, 2 min with Milli-Q water and 2 min with buffer. For the chemically modified capillaries, the rinse consisted of 3 min. with the buffer.

Instrumentation

HPCE experiments were done at 30°C on a Perkin-Elmer/Applied Biosystems Model 270A-HT Capillary Electrophoresis System. All injections were made hydrodynamically. Detection was at 223 nm.

Sample Preparation

Melatonin, purchased from a pharmacy in tablet form, was ground to a fine powder. One milligram of the ground material was dissolved in 10 mL of pH 4.41 buffer and sonicated for 15 min. This solution was injected into the CE apparatus directly.

RESULTS AND DISCUSSION

Analysis of Melatonin Only

One potential analytical problem involves the analysis of melatonin in the presence of tryptamine and serotonin without the need for either a qualitative or quantitative determination of the latter two compounds. In this case it is only necessary to ensure that there is adequate separation between the first two components and melatonin. The conditions for such a determination, shown in Figure 2, involve the use of a bare capillary and a running buffer at pH = 4.41.

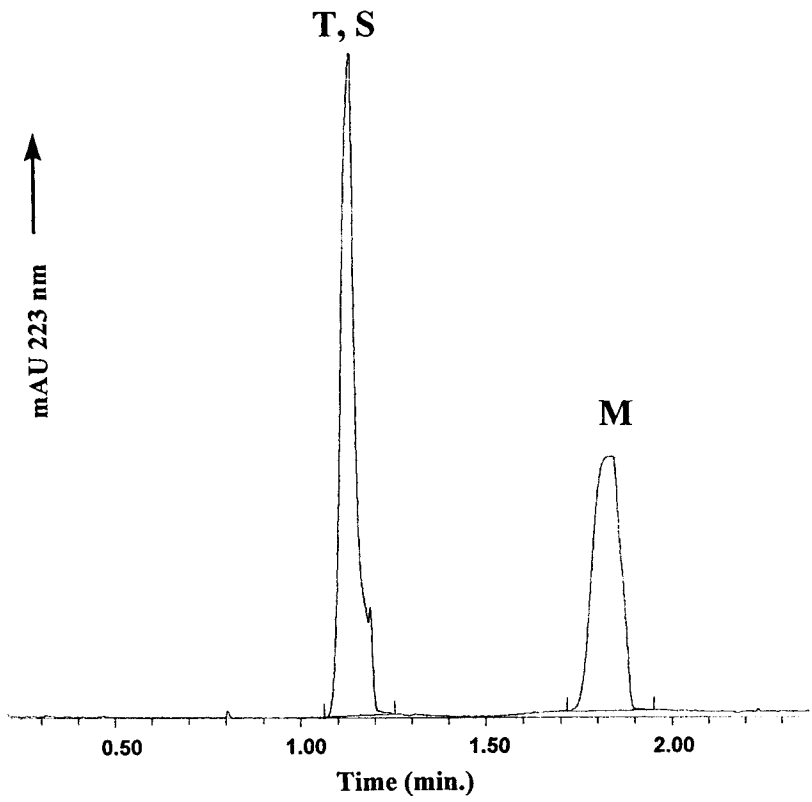


Figure 2. Electropherogram of mixture on bare capillary at pH =4.41. Column: 75 μm fused silica, L = 50 cm, l = 25 cm, V = 30 kV, I = 24 μA. Injection for 2 s at 12.6 cm Hg vacuum (1.68×10^4 Pa). Solutes: T = tryptamine, S = serotonin, M = melatonin.

The first peak includes both tryptamine and serotonin which are unresolved but with good separation from the melatonin peak. The total analysis time is about two minutes which is an order of magnitude faster than the normal phase HPLC method with high detection efficiency.[8]

The linear range for good quantitative measurements of melatonin is illustrated in Figure 3. The complete range (Fig. 3A) encompasses concentrations from 250 ng/mL up to about 10 μg/mL. The lower part of the linear dynamic range is shown in Fig. 3B. Detection below 250 ng/mL is possible with the lower limit being about 25 ng/mL but reproducibility is generally > ± 10%. The quantitative measurements were made in a 75 μm capillary to improve sensitivity but ordinary UV detection was used.

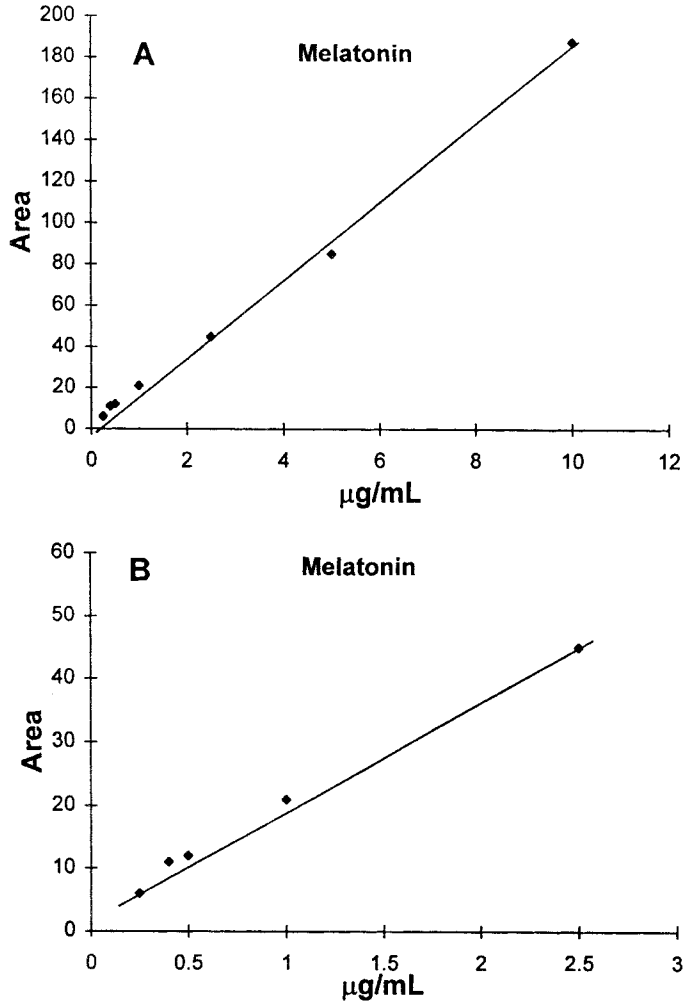


Figure 3. Calibration curve for analysis of melatonin. (A) Complete linear range and (B) Low end of linear dynamic range. Conditions for analysis described in Fig. 2.

The wavelength chosen for the analysis was 223 nm, the absorption maximum for melatonin. A more sensitive detection system could improve the lower limit of detection closer to that observed by the best HPLC method,[8] i.e. about 10 pg/mL. However, it should be noted that the 10 pg/mL level of detection was achieved after considerable sample preparation. As a practical test for quantitative measurements, a commercial melatonin tablet was analyzed

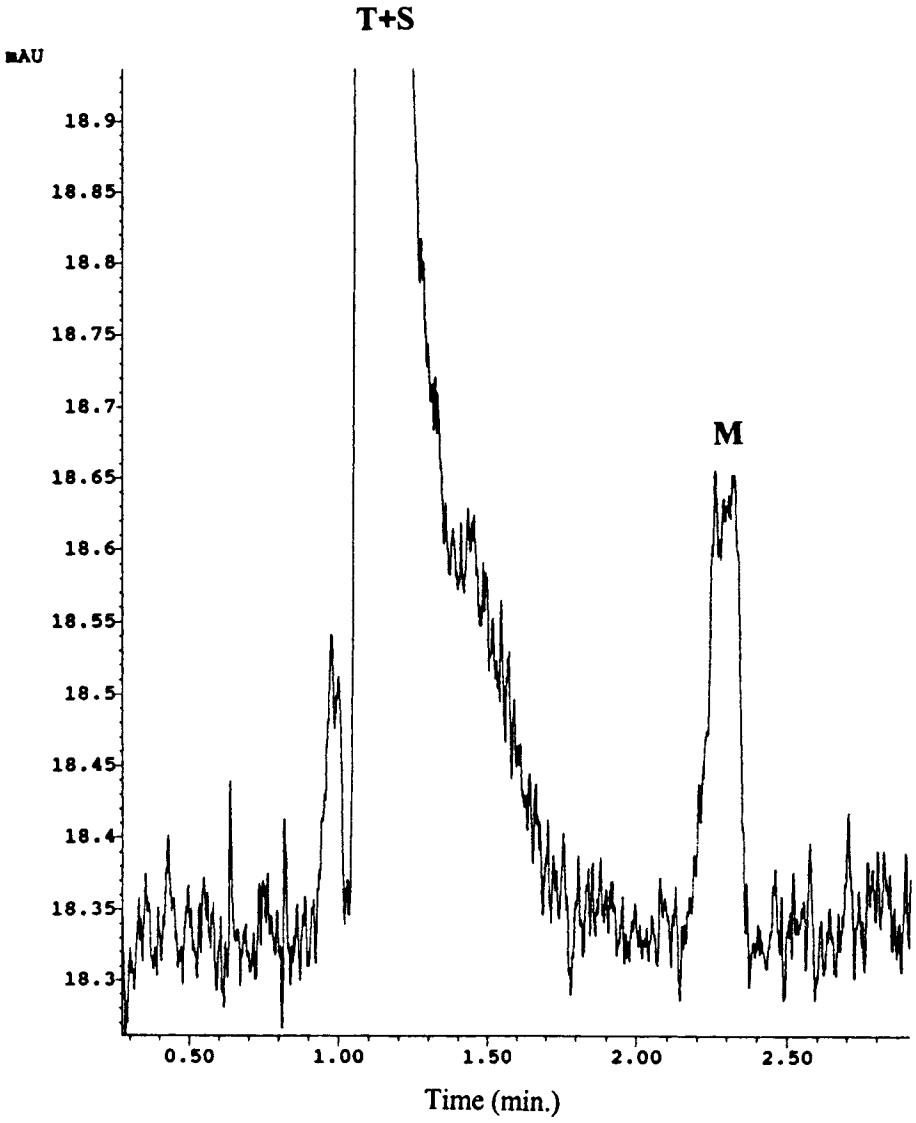


Figure 4. Electropherogram for analysis of melatonin in the presence of large excess of tryptamine and serotonin. Conditions for analysis described in Fig. 2. [T] = [S] = 10 μg/mL. [M] = 50 ng/mL.

using the conditions outlined above. The manufacturer's test data, determined by HPLC, reported 3 mg of melatonin per tablet. The HPCE method, based on five determinations, gave a value of 2.92 ± 0.08 mg/tablet or a relative error of ±2.7%.

One problem encountered in the HPLC methods is an adequate separation between melatonin and the other two components when the concentrations of tryptamine and serotonin can often be greater by a factor of 100. Figure 4 shows the electropherogram obtained for a mixture containing 10 μg/mL each of serotonin and tryptamine and 50 ng/mL of melatonin. Clearly large concentration differences are not a problem in the HPCE method so that the analysis of samples from physiological fluids or tissues after appropriate preparation steps should be possible.

Analysis of all Three Compounds

In order to separate all three compounds completely, it is necessary to lower the pH so that the electroosmotic flow will be reduced significantly in the bare capillary. It was found that a pH of 2.14 provides the desired separation as shown in Figure 5. Under these conditions, tryptamine and serotonin are fully charged but melatonin still remains almost neutral with a pK_a below 2. The electroosmotic flow is reduced significantly from pH = 4.41 so that tryptamine and serotonin can be separated by their differences in electrophoretic mobility. There is either sufficient charge on the melatonin or, electroosmotic flow (EOF) present so that serotonin has a migration time of just under twenty minutes. Quantitation of melatonin with results comparable to those obtained at pH 4.41 is possible using a 75 μm capillary. Tryptamine and serotonin can be determined individually with good accuracy in the range of 500 ng/mL to 5 μg/mL.

Analysis of only Tryptamine and Serotonin

In order to obtain better separation of tryptamine and serotonin, a capillary with very low electroosmotic flow is necessary. A very low EOF can be best achieved by modifying the capillary walls in order to derivatize as many of the residual silanols as possible. Some examples of this separation on three modified surfaces are shown in Figure 6. The AEG and C_{18} moieties are attached to the silica surface of the capillary wall by first converting the surface silanols to hydrides via a silanization reaction and then attaching the organic group via hydrosilation[19,20] while the diol column is made by first etching the surface and then attaching the organic species by the same reaction protocol.[13,20] In all three cases the results are similar.

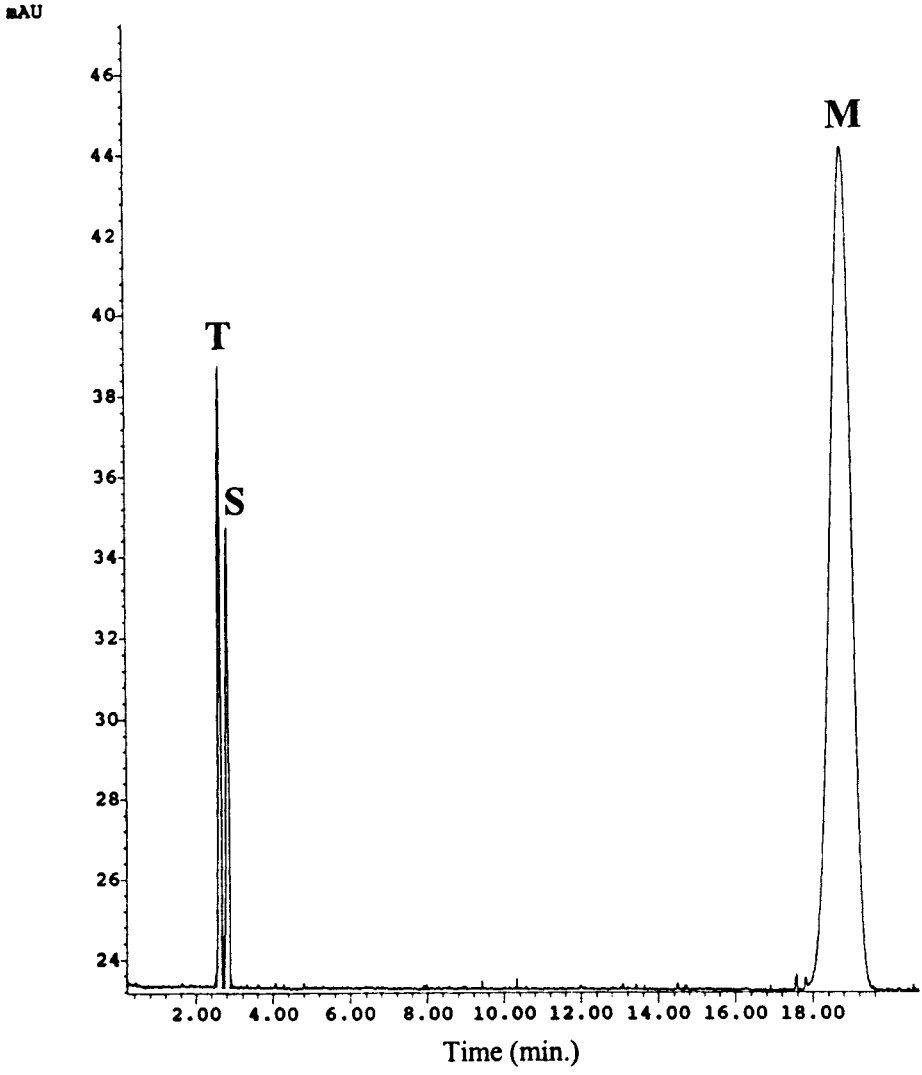


Figure 5. Electropherogram of mixture on bare capillary at pH = 2.14. All conditions are the same as in Fig. 2 except I = 57 μA.

The EOF has been lowered enough so that the migration through the column is slower than with a bare capillary and better separation of the compounds occurs. The resolution is also improved by the use of sample stacking which produces a much narrower band width.

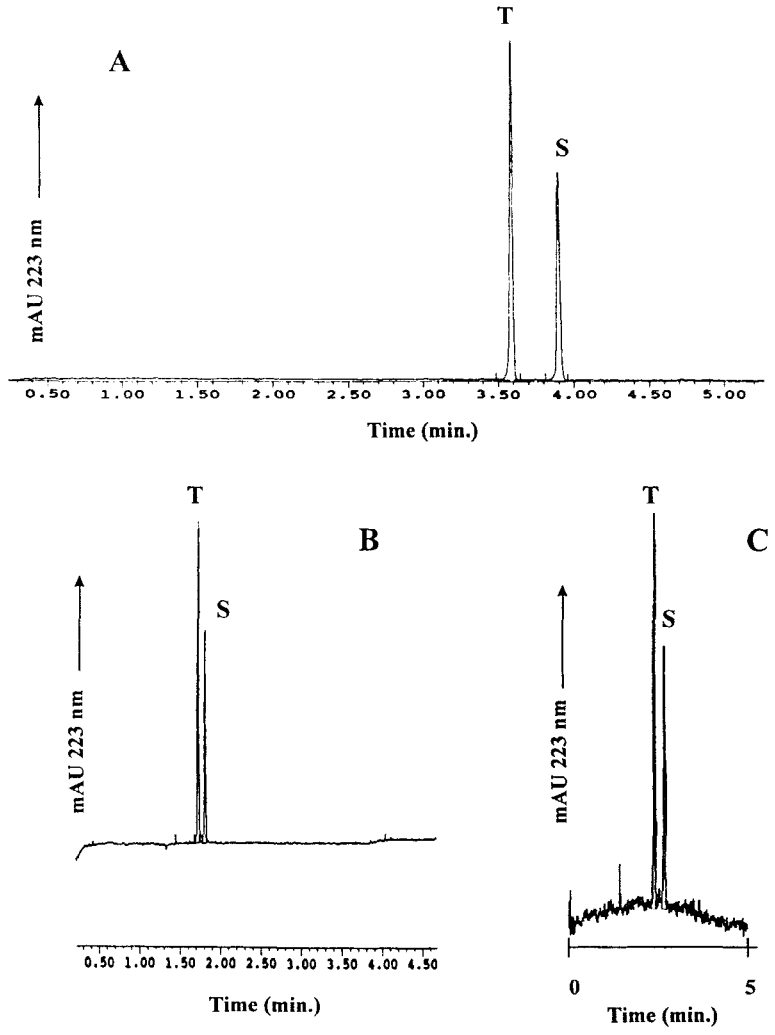


Figure 6. Electropherograms of mixture on chemically modified capillaries. (A) AEG capillary: 75 μm, L = 50 cm, l = 25 cm, pH 2.14 buffer, V = 30 kV, I = 57 μA, injection 2 s at 12.6 cm Hg vacuum (1.68×10^4 Pa), sample in water. (B) C-18 capillary: 50 μm, L = 50, l = 25, pH 4.41 buffer, V = 30 kV, I = 13 μA, injection 3 s at 12.6 cm Hg, sample in water. (C) Diol etched capillary: 50 μm, L = 50 cm, l = 25 cm, pH 2.14 buffer, V = 30 kV, I = 56 μA, injection 3 s at 12.6cm Hg, sample in water.

Quantitation of serotonin and tryptamine is much easier under these conditions since there is no band overlap. Good quantitation is possible in the range of 250 ng/mL to approximately 10 μg/mL. The detection limit for both compounds is about 100 ng/mL. The linear range and detection limits are easily compatible with the level of serotonin found in serum samples which is typically around 3 μg/mL.[21] However, under the conditions of low EOF the negligibly charged melatonin takes more than 40 min to migrate through the column making analysis for the three component mixture impractical. From the separations shown in Figure 6, it does not appear that CEC utilizing the etched modified capillaries offers any advantage over normal capillaries which have been modified with an organic moiety. The two HPCE capillaries and the CEC capillary give comparable separations with some differences in migration times, which probably reflects the number of residual silanols on the surface.

CONCLUSIONS

HPCE offers a viable alternative to HPLC for the analysis of melatonin, serotonin, and tryptamine. The analysis times are generally faster with sensitivity using an ordinary UV detector acceptable for many analyses. Tryptamine and serotonin are well separated from melatonin so that analysis of the latter in the presence of a large excess of the other two compounds is quite easy. By appropriate choice of capillary and buffer, an analysis for any one or all three components can be accomplished rapidly and efficiently. HPCE can be directly adapted to quality control of melatonin in pharmaceutical samples. The assay of biological fluid and tissue samples was beyond the scope of this investigation. However, the preliminary results obtained indicate that after appropriate sample preparation step(s), the HPCE and CEC protocols described here could be just as adaptable to biological analyses as HPLC.

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