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QUANTITATIVE DECONVOLUTION OF HEAVILY FUSED CHROMATO-GRAPHIC PEAKS OF BIOLOGICAL COMPONENTS USING A MULTI-WAVELENGTH UV DETECTOR

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SUMMARY

Quantitative deconvolution of a chromatographic peak with extremely low UV absorption (less than 0.005 A.U.) is demonstrated for the analysis of an anaesthetic (ketamine) in rabbit serum. One ketamine metabolite, nor-ketamine, was deconvoluted from a completely fused peak in the three-dimensional chromatogram by using a highly sensitive multi-wavelength UV detector. After injection of ketamine, the nor-ketamine level in the serum increased to 3 μg/ml, calculated as ketamine, in 120 min.

INTRODUCTION

When a chromatogram is monitored with a spectrophotometric detector, it should be defined as a series of spectra as a function of time. However, a chromatogram must be regarded as a cross-section of a series of spectra along the time axis, owing to lack of suitable instrumentation.

Earlier, attempts were made to use an ordinary spectrophotometer to obtain a spectrum of a sample component in a chromatographic peak by using the stopped-flow technique. However, it was necessary to interrupt the chromatographic elution to obtain the spectra. Chromatography is, in principle, a time-dependent phenomenon and, therefore, such a technique failed to be accepted by chromatographers.

Denton *et al.*[1] reported the use of an oscillating mirror rapid-scanning spectrophotometer as a high-performance liquid chromatographic (HPLC) detector, capable of generating a three-dimensional chromatogram. However, even though the flow cell was as large as 87 μl, the noise levels below 250 nm were fairly high (±0.05 to ±0.2 A.U.)[2]. As micro-particulate packing materials capable of yielding narrow and fast peaks became available, such instrumentation rapidly became obsolete.

An instrument that incorporated a vidicon detector[3] was reported as an alternative, but this was soon replaced by a solid-state linear photodiode-array detector[4-6]. The use of a photodiode array improved the sensitivity owing to improvements in the efficiency of light energy utilization, because the detector is always irradiated. However, a self-scan type of photodiode array hardly allows real symmetrical double-beam optical arrangement, which results in poor long-term stability. In addition, a current-photodiode array with 128, 256 or 1024 elements does not properly match the available optics, because the 25 μm element spacing interval[7] is too narrow to be used with the entrance slit of a polychromator. If one attempts to obtain the maximum spectral resolution available, in general the image of the cell hole should be focused on the entrance slit of width 25 μm, resulting in extremely poor sensitivity owing to a significant loss of energy on introduction of the light into the polychromator. Therefore, in practice, in order to obtain the necessary sensitivity, outputs of several elements are summed and treated as a single output, which also meets the realistic slit width suitable for collecting light efficiently from the flow cell[4,8].

Recently, we reported a multi-wavelength UV detector incorporating symmetrical double-beam optics and a 32-element discrete-photodiode array, the element width of which is ideally suited to a 1-mm I.D. flow-cell[9,10]. This configuration achieved a noise level of less than $5 \cdot 10^{-5}$ A.U. at 250 nm, $1 \cdot 10^{-4}$ A.U. for the entire wavelength range from 195 to 350 nm and a linear dynamic range up to 1.8 A.U.

Three-dimensional chromatographic data, acquired by a multi-wavelength UV detector, can be manipulated by computer-aided techniques for graphical presention in various fashions, such as three-dimensional, contour and ratio chromatograms. Another aspect of computer-aided techniques is the peak deconvolution of unresolved chromatographic peaks by the use of standard reference spectra[3,9,11-18]. Applications of the peak deconvolution technique have so far been limited to very simple and known components, and practical applications to multi-component samples, such as physiological fluids, have not yet been possible. The reasons that prevent the technique from being extended to such samples include the following shortcomings of existing instruments: (1) low signal-to-noise ratio, which distorts portions of the spectrum having weak absorbance; (2) limited linear dynamic range, which also distorts portions of the spectrum having strong absorbance; and (3) poor stability of the chromatographic and spectral baselines, which distorts the overall shape of the spectrum. The spectral distortion caused by these shortcomings would not permit the peak deconvolution to be carried out at the high and low ends of the sensitivity range.

In order to investigate the applicability of peak deconvolution, it was applied to a peak with extremely low UV absorption (less than 0.005 A.U.) by using a newly developed multi-wavelength UV detector in the analysis of an anaesthetic (ketamine) and its metabolite (nor-ketamine) in rabbit serum.

EXPERIMENTAL

Reagents

Ketamine (Ketalar 50) was obtained from Sankyo (Japan). Methanol and acetonitrile were of LC grade, purchased from Wako (Osaka, Japan).

Nor-ketamine from culture fluid of ketamine with isolated rat hepatocytes was used as a reference[19].

Instruments

A MULTI-320 multi-wavelength UV detector (JASCO, Tokyo, Japan) and a DS-L800 data processor were used with a JASCO TRI ROTAR-VI HPLC system.

Procedure

Ketalar 50 (700 μl) was injected into a rabbit at a level of 10 mg/kg as ketamine, which is equivalent to the dose for human subjects. About 2 ml of blood were drawn at intervals of 3 min for the first 30 min, then the intervals were increased as time elapsed. Each blood sample was centrifuged at 2800 g for 10 min. A 0.5-ml volume of the supernatant was added to 1.0 ml of methanol, then the mixture was centrifuged again under the same conditions. A 50-μl volume of the last supernatant was injected into a JASCO Fine Pak SIL C_{18} column (125 $\times$ 4.6 mm I.D.), which was eluted with acetonitrile–water (30:70) containing 60 mM perchloric acid.

Three-dimensional chromatographic data were acquired by the multi-wavelength detector. A chromatographic peak in which nor-ketamine was fused with another compound was quantitatively deconvoluted into the nor-ketamine peak and a peak originating from the serum. This procedure was performed for each serum and the nor-ketamine concentration obtained was plotted against time.

RESULTS AND DISCUSSION

Chromatographic and spectrometric identification of ketamine and nor-ketamine

In order to examine the retention times of ketamine and nor-ketamine, the ketamine standard and the culture fluid were chromatographed prior to the serum sample.
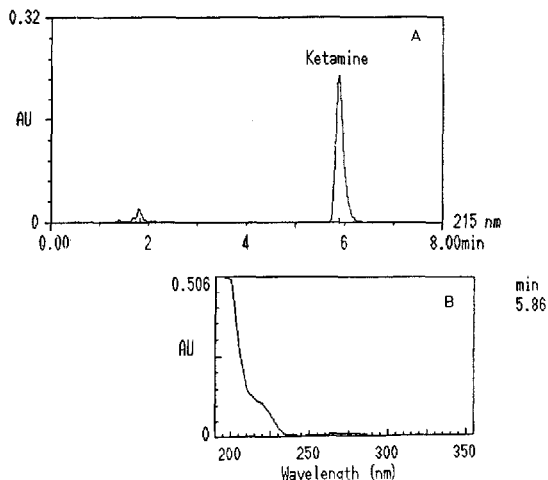

Fig. 1. Chromatogram and spectrum of the ketamine standard. (A) Chromatogram of the ketamine standard, monitored at 215 nm, obtained from the three-dimensional chromatographic data. A 2-μg amount of ketamine was eluted on a JASCO Fine Pak SIL C_{18} (125 $\times$ 4.6 mm I.D.) at 5.86 min. (B) UV spectrum taken at the peak. The ketamine standard spectrum has a small shoulder at 220 nm and a broad peak at 275 nm. Eluent: acetonitrile–water (30:70) containing 60 mM perchloric acid.
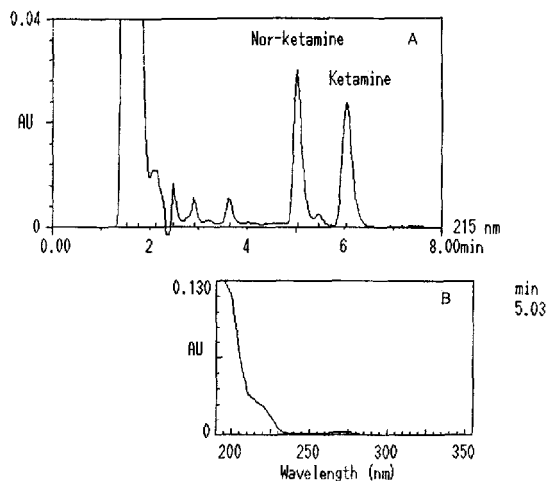
Fig. 2. Chromatogram and spectra of ketamine and nor-ketamine from the culture fluid. Nor-ketamine was eluted at 5.03 min, and its UV spectrum (B), taken at the peak, is also shown. The nor-ketamine spectrum is identical with that of ketamine.

Fig. 1A shows the chromatogram of the ketamine standard monitored at 215 nm obtained from the three-dimensional chromatographic data by the data reduction technique using the data processor. A 2-μg amount of ketamine was eluted at 5.86 min, and its UV spectrum (Fig. 2B), taken at the peak, is also shown. The ketamine spectrum has a small shoulder at 220 nm and a broad peak at 275 nm.

Fig. 2A shows the chromatogram of ketamine and nor-ketamine from the culture fluid. Nor-ketamine was eluted at 5.03 min; its UV spectrum (Fig. 2B), taken at the peak, is also shown. The nor-ketamine spectrum is identical with that of ketamine.

Fig. 3 shows the three-dimensional chromatogram of rabbit serum taken 18 min after the administration of ketamine. A 50-μl volume of the pretreated serum was separated with the same chromatographic system as before. As it is already known that nor-ketamine and ketamine would be eluted at 5.03 and 5.86 min, respectively, peaks were sought near these retention times with spectra equivalent to that of the ketamine standard. It was found that the peak eluted at 5.98 min had a
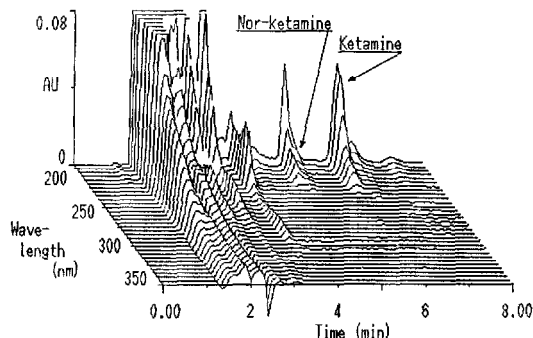

Fig. 3. Three-dimensional chromatogram of rabbit serum taken 18 min after ketamine administration. A 50-μl volume of pretreated serum was separated with the same chromatographic system as before.
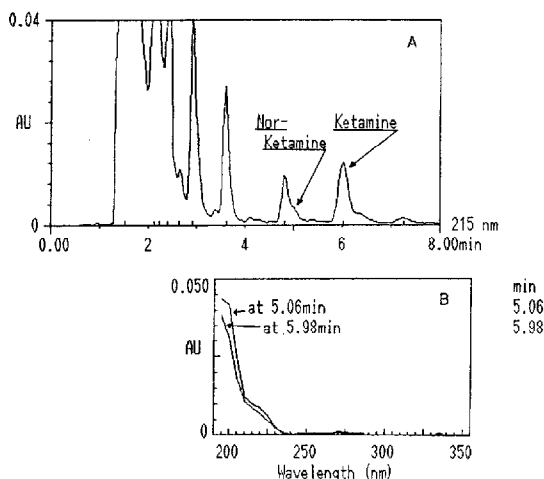
Fig. 4. Chromatogram and spectra of rabbit serum. (A) Chromatogram monitored at 215 nm and (B) spectra taken at 5.06 and 5.98 min. The spectra were obtained by subtracting the spectra taken just before the rise of the 5.06 min peak and the fall point of the 5.98 min peak from the spectra at those times. The 5.98 min peak had a spectrum that agreed with the ketamine standard spectrum, and the portion at 5.06 min of the peak that appeared as a tail also had the same spectrum.

spectrum that agreed with that of the ketamine standard, and a portion of the peak which appeared as a tail at 5.06 min had also the same spectrum.

Fig. 4 shows (A) a chromatogram monitored at 215 nm and (B) spectra taken at 5.06 and 5.98 min, which were obtained from the same chromatographic data. The spectra were obtained by subtracting the spectra taken just before the rise of the 5.06 min peak and the fall of the 5.98 min peak from the spectra at those times in order to eliminate the backgrounds. These spectra were obtained accurately enough to be compared with the standard spectrum. Fig. 5 shows the correlation between a spectrum taken at 5.06 min and the nor-ketamine reference spectrum taken from the culture fluid analysis. The two spectra were in good correlation ($\gamma = 0.994$). These
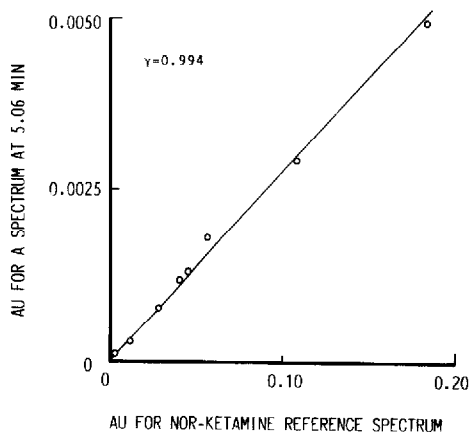

Fig. 5. Correlation between the spectrum at 5.06 min and the nor-ketamine reference peak.
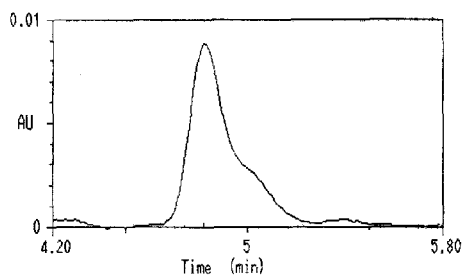
Fig. 6. Observed peak, including nor-ketamine. An enlargement of an observed peak that includes nor-ketamine, is shown.

components did not appear in the chromatogram of the control serum, taken just before the administration of ketamine. However, they were present in those of the sera after administration. In addition, their amounts varied with time. Hence they were confirmed to originate from ketamine. Therefore, the two peaks were chromatographically and spectrometrically identified to be nor-ketamine and ketamine, respectively.

Quantitative deconvolution of the nor-ketamine peak

Fig. 6 shows an enlargement of an observed peak that includes nor-ketamine. It was completely fused into the tailing part of the main peak, which originated from the serum, so that a conventional peak integration method failed to quantitate it. Therefore, peak deconvolution, in which the least-squares method is utilized[10], was performed on the spectral range from 210 to 300 nm and the time range from 4.2 to 5.8 min, using the nor-ketamine reference spectrum and the spectrum of the peak with which nor-ketamine peak is fused. The latter spectrum was obtained from the data for the control serum, taken just before administration of ketamine.

The observed peak 1 was deconvoluted into two peaks, 2 and 3, as shown in Fig. 7. Peak 3 represents the fraction that agrees with the nor-ketamine spectrum. Peak 2 is a fraction originiting from the serum. The heavy line shows a residual curve, that is, the square root of the sum of the squares of differences between the observed spectrum and the re-convoluted spectrum from the spectra of peaks 2 and 3. That
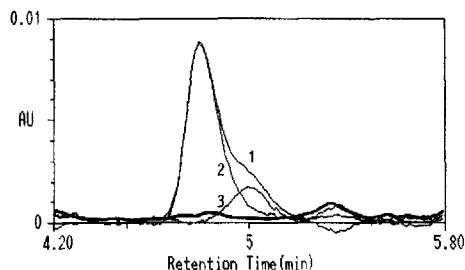

Fig. 7. Quantitative deconvolution of nor-ketamine peak. The peak deconvolution was performed by using the spectrum of the nor-ketamine standard and that of the peak in which nor-ketamine peak is fused. The observed peak 1 was deconvoluted into two peaks, 2 and 3. Peak 3 represents the fraction that agrees with the nor-ketamine spectrum. Peak 2 is a fraction originating from the serum. The heavy line shows the residual curve that indicates the performance of the peak deconvolution (see text).
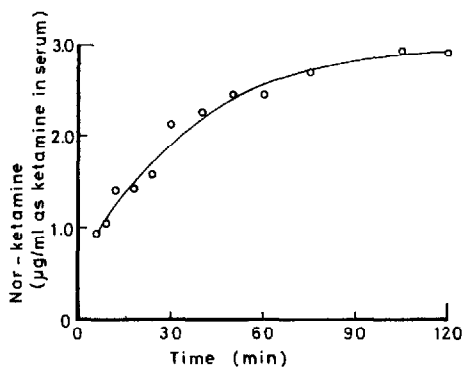
Fig. 8. Time course of nor-ketamine levels. Even though the peak was completely fused and weak, quantitation was carried out successfully. The time course shows a reasonable curve for a ketamine metabolite. After ketamine injection, the amount of nor-ketamine increased to a level of 3 ± 0.3 μg/ml, calculated as ketamine.

is a good indication of the performance of the peak deconvolution. For the quantitation of nor-ketamine, peak 3 was integrated from 4.70 to 5.20 min. The level of the residual curve remained below $5 \cdot 10^{-4}$ A.U. in this time range. The small peak at 5.34 min on the residual curve represents a component that does not fit the spectra used in the deconvolution.

Fig. 8 shows the time course of nor-ketamine levels in the serum obtained by peak deconvolution. Even though the peak was completely fused and the spectral peak was weak, quantitation was carried out succesfully, as shown. The time course is a reasonable curve for a ketamine metabolite. After ketamine injection, the level of nor-ketamine increased to 3 ± 0.3 μg/ml, calculated as ketamine, in 120 min.

CONCLUSION

A multi-wavelength detection system generally allows the quantitative analysis of chromatographic peaks that include unresolved components by using the peak deconvolution technique. However, the utility of the technique is determined by the performance of the detection system itself, *i.e.*, the attainable precision of the spectral data, rather than the sophistication of the data processor. In general, more data points give a more accurate spectrum, but this is true only when the noise level and the linear dynamic range are independent of the number of data points, in other words, the spectral band width. Further, in practice, excessive amounts of data require excessive processing time or the use of an expensive large computer. Therefore, as a design criterion for a practical multi-wavelength UV detector, there should be good balance between the spectral band width, sensitivity, linear dynamic range, accuracy and precision of spectral data.

These successful results of the application of peak deconvolution in biological component analysis show that a band width of 5 nm, a noise level of $1 \cdot 10^{-4}$, a linear dynamic range up to 1.8 A.U. and a wavelength range of 195–350 nm represent a good example of a well designed system for practical applications.

REFERENCES

1 M. S. Denton, T. P. DeAngelis, A. M. Yacynych, W. R. Heineman and T. W. Gilbert, *Anal. Chem.*, 48 (1976) 20.
2 M. J. Milano, S. Lam and E. Grushka, *J. Chromatogr.*, 125 (1975) 315.
3 A. E. McDowell and H. L. Pardue, *Anal. Chem.*, 49 (1977) 1171.
4 R. E. Dessy, W. D. Reynolds, W. G. Nunn, C. A. Titus and G. F. Moler, *J. Chromatogr.*, 126 (1976) 347.
5 Y. Talmi, *Anal. Chem.*, 47 (1975) 697A.
6 Y. Talmi, *Anal. Chem.*, 47 (1975) 658A.
7 *Product Summary, Solid State Image Sensors*, 055-0014 38003 EG&G RETICON, Sunnyvale, CA.
8 S. A. George, *Pittsburgh Conference, 1984, Abstr. No. 175.*
9 S. Tohei, S. Kohara and M. Saito, *Pittsburgh Conference, 1984, Abstr. No. 179.*
10 T. Hoshino, M. Senda, T. Hondo, M. Saito and S. Tohei, *J. Chromatogr.*, 316 (1984) 473.
11 R. G. Berg, C. Y. Ko, J. M. Clemons and H. M. NcNair, *Anal. Chem.*, 47 (1975) 2480.
12 M. J. Milano, S. Lam, M. Savonis, D. B. Pautler, J. W. Pav and E. Grushka, *J. Chromatogr.*, 149 (1978) 599.
13 L. N. Klatt, *J. Chromatogr. Sci.*, 17 (1979) 225.
14 J. M. Halket, *J. Chromatogr.*, 175 (1979) 229.
15 M. L. Malczewski and E. Grushka, *J. Chromatogr. Sci.*, 19 (1981) 187.
16 M. McCue and E. R. Malinowski, *J. Chromatogr. Sci.*, 21 (1983) 229.
17 A. F. Fell, H. P. Scott, R. Gill and A. C. Moffat, *J. Chromatogr.*, 282 (1983) 123.
18 A. F. Fell, B. J. Clark and H. P. Scott, *J. Chromatogr.*, 316 (1984) 423.
19 T. Hoshino and H. Takahagi, *J. Chromatogr.*, submitted for publication.