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Standardization of a multi-wavelength UV detector for liquid chromatography-based toxicological analysis

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ABSTRACT

The performance of a multi-wavelength UV detector for automated drug identification following liquid chromatographic separation was evaluated. The ability of selected wavelength ratios to distinguish two closely related drugs was considered at different concentrations. Calibration of the detector based on wavelength ratios was then utilized to standardize two different detectors and to evaluate instrument-to-instrument variation of a series of detectors. Reproducibility of the second-derivative zero intercept for these drug spectra was also evaluated. Standardization of detector performance by reference to these two parameters permitted the transfer of UV spectral libraries stored on one instrument to another without compromising the reliability of qualitative data.

INTRODUCTION

The use of multi-wavelength detectors (MWDs) in liquid chromatographic (LC) systems was actively explored 15 years ago [1–4] and their use in analysis of drug mixtures has been demonstrated [5]. The introduction of commercial instruments within the last decade [6–8] has permitted numerous investigations, primarily in the fields of pharmaceutical analysis and analytical toxicology.

Evaluation of peak purity has emerged as the most common application of LC–MWD, owing to regulatory requirements governing drug purity and stability testing. It is not uncommon that impurities and breakdown products have near-identical retention times and spectra, compared with the parent. Three different techniques have been used to validate peak homogeneity: (1) wavelength ratios, either one or several ratios; (2) multi-point spectral comparison, using either normalized spectra or transformed data; and (3) evaluation of spectral derivatives, especially the second derivative.

Numerous evaluations of the usage and comparative utility of these approaches have been published [9–14]. In most instances, the ability of the technique to detect impurities of 1% or less has been shown.

MWDs have also been used to monitor the location of peaks during mobile

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phase optimization experiments. The three techniques listed above can be successfully employed for this purpose [15,16], but is is also possible to utilize a stored spectral library for peak matching, as the spectra of all components are known [16,17]. Multi-parameter techniques have also been employed [18].

In contrast to these applications, the use of LC–MWD for the qualitative analysis of toxicological samples always requires the use of a spectral library. Although dual-wavelength ratios have been applied with moderate success [19,20], increased computerization has permitted the development of a variety of algorithms for drug identification. The most straightforward approaches rely on only one parameter (in addition to the retention time). The fraction of total absorbance at λ_{max} (FTA) [21,22] has been proposed, in addition to a normal vector produced by a Fourier transform [23]. However, for forensic work an algorithm which utilizes multiple parameters is desirable for increased confidence in the degree of matching between a library and unknown spectra. The use of multi-parameter identification can be compared with the use of multiple ion ratios (with retention data) in gas chromatographic–mass spectrometric (GC–MS) identification of drugs [24] and the use of multiple solvent systems in thin-layer chromatography [25]. Accordingly, many investigators have reported multi-parameter identification schemes, combining wavelength ratios, spectral maxima and minima and comparisons of normalized spectra or other transformed data [26–31].

The widespread availability of an LC toxicology system would provide a useful supplement to existing screening methods. This has not been possible for several reasons. First, the succesful application of library searching algorithms has been demonstrated [20,22,29,32,33] but each of the investigators developed an in-house spectral library. No reports have demonstrated the successful transfer of stored spectral data from one chromatographic system to another. As common MWDs utilize a multi-element diode array, it is likely that spectra from different instruments will show variation. Difficulties associated with the transfer of GC–MS libraries have been reported previously [25,34]. Further, the robustness of search algorithms could be affected, at both high and low concentrations, by deviations of detectors from linearity. A recent report by Dose and Guichon [35] indicates that bias and non-linearity are commonly observed and are affected by both the shape of the spectrum and the detector bandwidth. Published validations of search algorithms have generally employed samples at only one concentration, generally with high absorbance.

We recently described a multi-column LC–MWD that permits on-line purification and reproducible separation of basic drugs [36]. In this work, the calibration of a scanning detector for that analytical system was investigated, using wavelength ratios and second derivatives to establish linearity and reproducibility. Finally, the calibration scheme was tested on a larger series of instruments, demonstrating the reproducibility of this approach.

EXPERIMENTAL

Apparatus

The computer-controlled multi-column apparatus has been described previously [36]. Briefly, urines or sera initially pass through two clean-up cartridges, where proteins, salts and hydrophobic acids and neutral endogenous compounds are re-

moved. A 2-ml fraction containing basic drugs reaches a coupled 25 × 3.2 mm I.D. reversed-phase cartridge (Shandon, Runcorn, UK) and 150 × 4.6 mm I.D. silica cartridge (Machery, Nagel & Co., Düren, Germany), where the analytical separation occurs. In this study, only the conditions for the final separation are of interest, so the system can be considered equivalent to an isocratic analysis using cation-exchange chromatography. The flow-rate was 1.5 ml/min and the temperature was maintained at 35°C.

In place of the Hewlett-Packard Model 1040A diode-array detector used in the previous work, we monitored absorbance with a Chrom-A-Scope detector (BarSpec, Rehovot, Israel). This detector utilizes a rotating holographic grating, which permits the collection of ten scans per second. After wavelength selection light passes through the flow cell to a single diode sensor. The collected analog data from the diode are digitized and integrated with the velocity and acceleration data from the scanning assembly to produce three-dimensional array consisting of time, wavelength and absorbance. The wavelength accuracy of the detector is specified as ± 1 nm, with repeatability better than 1 nm. Repeatability is maintained by monitoring a "null point" which is used to calibrate the grating drive mechanism with each rotation. Although it is a single-beam system, all spectra are corrected continuously for dark current. Also, all the spectra taken for each peak are corrected by subtraction of a background spectrum, collected after the conclusion of the peak.

For the experiments described below, the wavelength range was 193–305 nm and the spectral bandwidth was 5 nm. The baseline noise was less than 1 mAU at 205 nm. The flow cell had a 9-μl volume and 5-mm path length.

Software for calculation of wavelength ratios, similarity factor (*SF*) and second derivative zero intercepts (2DI) was written at BarSpec. Wavelength ratios were calculated from absorbance readings which were the average of five scans; thus each data point corresponded to a 0.5-s segment of the peak. All of the absorbance ratios from upslope mid-point to downslope mid-point of the chromatographic peak were considered in the determination of a median ratio (typically 16–24 values). Except for the lowest concentrations, the range of values about the median ratio was no more than ± 5%. Similarity factors were calculated after comparison of spectra normalized from 205 to 250 nm so that the maximum absorbance value for each spectrum was 1.00. The sum of squares of the normalized differences at each wavelength yielded a parameter (*SF*) which was typically 0.020 or less for repetitive injections of the same drug. An *SF* value greater than 0.150 is evidence of an unsatisfactory spectral match. The 2DI and *SF* were each determined at the apex of the chromatographic peak. For most of the drugs evaluated, more than one 2DI was observed, but only the value between 215 and 245 nm was considered.

Chemicals

J. T. Baker high-performance liquid chromatographic (HPLC)-grade acetonitrile was obtained from VWR (Brisbane, CA, USA); Each batch was checked for conformity with the manufacturer's absorbance specification (less than 0.02 absorbance units at 200 nm); HPLC-grade potassium dihydrogen phosphate was from Fisher (Santa Clara, CA, USA) and N,N-dimethyloctylamine from Aldrich (Milwauke, WI, USA). All other laboratory chemicals were of analytical-reagent grade. Water was purified using an in-house ion-exchange system and was equivalent to

HPLC grade. Drugs were obtained from Alltech (State College, PA, USA) or Sigma (St. Louis, MO, USA).

After preliminary washing and cleaning steps, drugs were eluted from the cation-exchange column with a mobile phase consisting of 6 m*M* phosphate buffer containing 2 m*M* dimethyloctylamine (adjusted to pH 6.35 with phosphoric acid)–acetonitrile (2:1, v/v).

A 1 g/l stock solution of each drug was prepared in methanol and stored at −20°C. Working solutions of the drugs (0.3–20 mg/l) were prepared by dilution with urine from healthy individuals receiving no medication.

RESULTS AND DISCUSSION

Identification of amphetamine abuse by analysis of urine samples requires the differentiation of this drug from several closely related analogues. Some of these compounds are available without prescription for diet control (*e.g.*, phenylpropanolamine) or as decongestants (*e.g.*, pseudoephedrine). Others have no established medical usage but are illegally distributed, including the 'designer drugs' (*e.g.*, methylenedioxyamphetamine).

We evaluated the properties of amphetamine and several related drugs using our chromatographic system (Table I). The parent compound, methamphetamine, elutes well after amphetamine and is readily distinguished from it, but it must in turn be differentiated from phendimetrazine and other analogues. All of these drugs have a λ_{max} near 209 nm, which is of no value for differentiation. It is evident that retention time data alone can readily distinguish amphetamine from ephedrine, a contaminant often seen in methamphetamine preparations. Methoxyamphetamine and methylenedioxyamphetamine have spectra which are readily distinguished from amphetamine, as the *SF* value is well above our cut-off (0.150). Phenmetrazine, which is a secondary amine with a heterocyclic ring, has a spectrum that does not differ greatly from amphetamine (*SF* = 0.043) but has a different 2DI (221 *vs.* 223 nm). If the 2DI could be determined with high reproducibility, it would aid in the differentiation of phen-

TABLE I

RELATIVE RETENTION TIMES (RRT) AND SIMILARITY FACTORS (*SF*) FOR AMPHETAMINE AND RELATED DRUGS

Similarity factors were calculated by comparison with amphetamine. The internal standard was chlorpheniramine. For retention data, each drug was analyzed five times over a 4-month period.

Compound	RRT ± S.D.	2DI	*SF*
Phenylpropanolamine	0.298 ± 0.008	223	0.049
Chlorphentermine	0.320 ± 0.010	235	8.747
Methylenedioxyamphetamine	0.327 ± 0.013	227	3.505
Methoxyamphetamine	0.335 ± 0.009	241	12.156
Phentermine	0.343 ± 0.007	223	0.007
Amphetamine	0.349 ± 0.013	223	–
Phenmetrazine	0.384 ± 0.005	221	0.043
Ephedrine	0.385 ± 0.007	223	0.001
Pseudoephedrine	0.402 ± 0.008	223	0.002

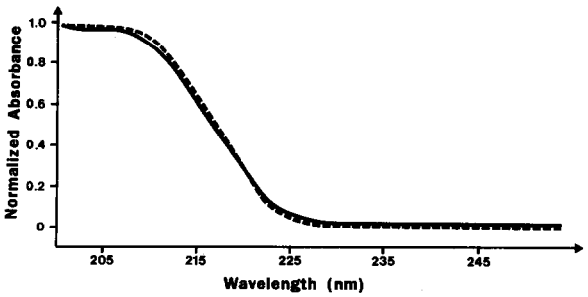

Fig. 1. Normalized spectra of (solid line) amphetamine and (dashed line) phentermine. For the data shown, $SF = 0.007$.

metrazine from amphetamine, ephedrine and pseudoephedrine. Finally, phentermine (α,α-dimethylphenethylamine) differs by only one α-methyl group from amphetamine (α-methylphenethylamine); the two compounds have very similar spectra (Fig. 1) and cannot be distinguished on the basis of relative retention times, 2DI or *SF*.

The very slight difference in the spectra in Fig. 1 was further evaluated as a test of detector reproducibility. The wavelength ratios at 5 nm intervals were calculated after duplicate analysis of each drug (Table II). The maximum difference in wavelength ratios (about 7%) was observed from 211:216 to 215: 220 nm. Although the largest differences were obtained at the highest wavelength ratio, the actual absorbances were less, which would limit sensitiviy. On the other hand, the baseline noise and background due to mobile phase increases at lower wavelengths. The 213:218 nm ratio was chosen as a compromise; the absorbance at 218 nm is about 50% of the maximum absorbance at 209 nm.

TABLE II

EVALUATION OF DIFFERENCES FOR WAVELENGTH RATIOS OF AMPHETAMINE AND PHENTERMINE

5 mg/l solutions were analyzed in duplicate.

Wavelength ratio (nm)	Phentermine	Amphetamine	Difference (%)
200:205	1.075	1.038	3.4
201:206	1.018	1.012	0.6
202:207	1.002	1.000	0.2
203:208	1.002	1.008	−0.6
204:209	1.014	1.025	−1.1
205:210	1.020	1.046	−2.5
206:211	1.069	1.089	−1.8
207:212	1.114	1.142	−2.4
208:213	1.169	1.210	−3.4
209:214	1.235	1.282	−3.7
210:215	1.313	1.385	−5.2
211:216	1.392	1.489	−6.5
212:217	1.499	1.601	−6.4
213:218	1.624	1.744	−6.9
214:219	1.796	1.942	−7.5
215:220	1.996	2.151	−7.2

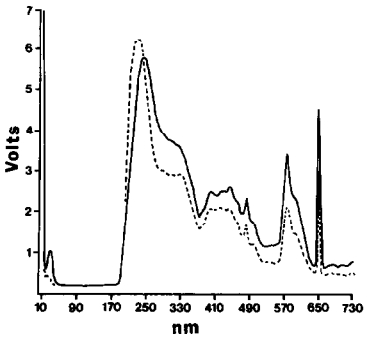

Fig. 2. Plot of the output of the deuterium lamp, collected in two different detectors. Solid line, unit 1; dashed line, unit 2.

Next, the ratio studies were used to evaluate the effect of calibration on detector performance. The detector manufacturer provides software for wavelength calibration, based on the output of the deuterium lamp. Although the maximum output is near 235 nm, this peak is a very broad line (Fig. 2). Further, exact location of this line may be affected by the characteristics of the quartz flow cell, the diode sensor and the aluminized mirror, which have different transmittance features. In contrast, the line at 651 nm is very sharp. A sharp emission peak is of little value for detection purposes, but is very useful for calibration. Therefore, the autocalibration software assigns 651 nm to this peak, with a stated accuracy of ± 1 nm. It may be observed in Fig. 2 that calibration on the 651-nm line is straightforward and that the observed lamp output from 200 to 250 nm showed variation between the two detectors.

The operating software permits reassignment of the calibration peak. The effect of this type of adjustment is shown in Table III. The 205:209 and 209:213 nm are shown for comparison. A shift of even 1 nm has a significant effect on all detection parameters. The 213:218 nm ratio is located at a point on the curve where absorbance changes rapidly, and it is profoundly affected by calibration. It is clear that any inaccuracy in calibration will alter this ratio.

To study variations between detectors, wavelength ratios for amphetamine and phentermine were collected using two different detectors, at concentrations that

TABLE III

RELATIONSHIP BETWEEN LAMP CALIBRATION AND AMPHETAMINE IDENTIFICATION CRITERIA

Calibration peak (nm)	Ratio 205:209 nm	Ratio 209:213 nm	Ratio 213:218 nm	2DI	Similarity factor, 205:250 nm
649	1.112	1.371	2.307	221	0.316
650	1.071	1.291	2.083	221	0.116
651	1.041	1.221	1.885	223	0.015
652	0.997	1.114	1.570	225	0.143
653	0.996	1.070	1.436	225	0.400

TABLE IV

WAVELENGTH RATIOS FOR TWO DIFFERENT DETECTOR UNITS

Each ratio is the mean of four values collected from analyses on four consecutive days.

Compound	Concentration (mg/l)	Unit 1			Unit 2		
		Ratio 205:209 nm	Ratio 209:213 nm	Ratio 213:218 nm	Ratio 205:209 nm	Ratio 209:213 nm	Ratio 213:218 nm
Amphetamine	20.0	1.035	1.228	1.906	1.020	1.202	1.828
	10.0	1.034	1.223	1.885	1.020	1.201	1.804
	5.0	1.035	1.220	1.871	1.026	1.198	1.802
	2.0	1.038	1.216	1.850	1.037	1.203	1.784
	1.0	1.045	1.206	1.872	1.067	1.197	1.786
	0.5	1.060	1.231	1.805	1.068	1.237	1.736
	0.3	1.074	1.195	1.854	1.057	1.267	1.811
	Mean	1.046	1.217	1.876	1.042	1.215	1.793
Phentermine	20.0	1.018	1.190	1.805	1.012	1.157	1.638
	10.0	1.020	1.184	1.736	1.014	1.156	1.615
	5.0	1.021	1.180	1.713	1.019	1.146	1.605
	2.0	1.022	1.172	1.671	1.065	1.144	1.612
	1.0	1.027	1.168	1.666	1.059	1.150	1.590
	0.5	1.037	1.170	1.666	1.101	1.203	1.630
	0.3	1.045	1.158	1.663	1.074	1.204	1.535
	Mean	1.027	1.175	1.703	1.049	1.166	1.604

ranged from 0.3 to 20 mg/l. The absorbance range covered by these concentrations was 10–600 mAU. Each drug was analyzed on four successive days at each concentration (Table IV).

All of the wavelength ratios showed slight changes with respect to changes in concentration. The 205:209 nm ratio decreased in response to an increase in concentration, whereas the 209:213 nm and 213:218 nm ratios increased. The instrument-to-instrument difference was 1–2% at 205:209 nm and 209:213 nm, but at 213:218 nm the mean amphetamine ratios differed by 4.6% and the mean phentermine ratios differed by 6.2%. As the previous study (Table III) had suggested that a 1-nm change in wavelength could produce on the average a 13% change in this ratio, the observed differences are consistent with very good agreement in wavelength accuracy between the detectors. On the other hand, the instrument-to-instrument variation is similar in magnitude to the amphetamine–phentermine differences observed in Table II. Overlap of the reported 213:218 nm ratios may be observed between amphetamine on unit 2 and phentermine on unit 1.

The within-instrument data for the 213:218 nm ratio for unit 1 are shown in Fig. 3. Although the mean ratios drift slightly downward and are slightly less precise at the lowest concentrations, the ± 2 S.D. ranges do not overlap at any concentration. Therefore, this ratio would be of considerable value in the differentiation of amphetamine and phentermine.

Several limitations of this technique for qualitative analysis should be apparent.

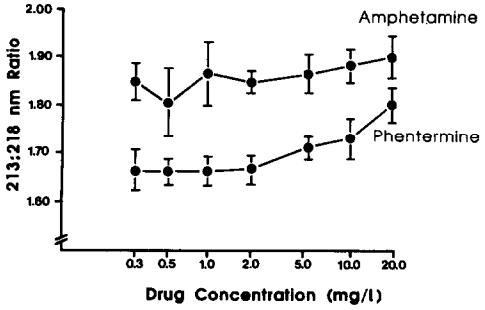

Fig. 3. Mean ± 2 S.D. ranges for the 213:218 nm ratio of amphetamine and phentermine ($n = 4$). The data were collected on four successive days on unit 1.

First, in a toxicological examination, the concentration of drugs is not known. In practice, solutions containing amphetamine and phentermine yielding peak heights similar to the unknown should be prepared and analyzed. Second, this approach could not be incorporated in any automated search routine owing to the instrument-to-instrument differences. The purpose of this investigation was to document a measurement that is highly reproducible on a single detector unit but showed variability between units and could be used for assessment of instrument differences.

From each of the analyses reported in Table IV, a corresponding set of 2DI data were collected (Table V). It may be observed that the reproducibility was very high. In no instance did the 2DI differ by more than 2 nm from 223 nm; the outlier values were seen at both high and low drug concentrations. We have confirmed this reproducibility in many other studies and propose that the location of 2DI can be used to corroborate small differences in normalized spectra (*SF*), *e.g.*, with phenmetrazine and ephedrine. Because 2DI is reported as an integer, any slight calibration difference between units 1 and 2 could not be detected; there was in fact no difference in the reported 2DI statistics for amphetamine.

We further tested the reproducibility of 213:218 nm ratios for amphetamine

TABLE V

SECOND-DERIVATIVE INDEX (2DI) DATA FOR TWO DIFFERENT DETECTOR UNITS

Concentrations and number of analyses as in Table IV.

Compound	Parameter	Unit 1	Unit 2
Amphetamine	Mean 2DI ± S.D.	222.93 ± 0.38	222.92 ± 0.39
	Lowest result	221	221
	Highest result	223	223
	223 nm as % of all results	96	96
Phentermine	Mean 2DI ± S.D.	222.93 ± 0.38	223.32 ± 0.90
	Lowest result	221	221
	Highest result	223	225
	223 nm as % of all results	96	75

TABLE VI

VARIATION IN AMPHETAMINE IDENTIFICATION PARAMETERS BETWEEN DETECTOR UNITS

Unit No.	Calibration peak (nm)	213:218 nm ratio	2DI
1	651	1.871	223
2	651	1.802	223
3	652	1.777	223
4	651	1.892	223
5	651	1.853	223
6	652	1.894	223
7	651	1.745	223
Mean ± S.D.		1.833 ± 0.059	223 ± 0

using five additional detectors (Table VI).Unit 2 was chosen as a reference system. If the ratio was not between 1.71 and 1.91, the location of the calibration peak was adjusted by 1 nm to achieve the desired result. This acceptance range would produce a difference of no more than 12% between any two detectors, which corresponds to less than 1 nm, according to Table III. For two of the additional five units, a calibration adjustment was required. After adjustment, the 2DI for amphetamine on these units was 223 nm and normalized spectra (*SF*) compared with unit 2 gave values of 0.015 or less. Additional drugs were analyzed and compared with library spectra collected on unit 2, including methamphetamine, imipramine, morphine, methadone and codeine. In all instances, excellent agreement was observed for normalized spectral data and 2DI.

Several investigators have proposed algorithms that can be used to calculate optimum wavelength ratios for distinguishing library entries [37,38]. Choosing ratios in a toxicological setting is not easy, as hundreds of drugs and metabolites may be encountered. It is more practical to recognize that different regions of the spectra can be used to characterize certain drugs [27] and employ tests that focus on that spectral region. In this study similarity factors for normalized spectra covering the range 205–250 nm were considered, because this range is generally useful for amphetamines and opiates. Although most drugs show increased absorbance between 200 and 205 nm, the deuterium energy from the lamp is reduced and the mobile phase absorbance is increased in this region, resulting in high background noise. Selection of a 2DI between 215 and 245 nm yielded a distinct value for each drug. These two features, coupled with retention time data, provide a powerful multi-parameter approach for library matching. Because each is determined at the peak apex, they are minimally affected by incomplete resolution from other peaks, and they are not concentration dependent. Wavelength ratios are highly characteristic for many drugs but do show some concentration dependence, in agreement with a published study [35]. On the other hand, a carefully chosen ratio may be useful for judging wavelength calibration.

The use of an isocratic separation, rather than a gradient, eliminates a major source of variability which affected several earlier studies. However, the composition of the isocratic mobile phase must be tightly controlled with respect to pH and organic solvent concentration. Very small pH changes can produce significant spectral shifts for phenothiazines, barbiturates and other drugs. In practice, the pH has

been maintained within 0.02 pH unit and the acetonitrile concentration has been controlled to within 0.5%.

CONCLUSIONS

We have compared the performance of two spectral parameters, the wavelength ratio at 213:218 nm and the zero intercept of the second derivative, for amphetamine and phentermine. Using a multi-wavelength detector that employs a rotating holographic grating and a single diode sensor, we have shown the high reproducibility of each measurement, with minimum dependence on concentration. Because each measurement was dramatically altered by a change in calibration, we were able to compare the agreement in wavelength accuracy between several detectors. After proper calibration, the observed differences in the 213:218 nm ratio were consistent with a difference of less than 1 nm between any two instruments. The agreement between normalized spectra collected on different instruments after this calibration was excellent.

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