Journal of Chromatography, 428 (1988) 369–376
Biomedical Applications
Elsevier Science Publishers B V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4216

Note

Screening for drugs in clinical toxicology by high-performance liquid chromatography: identification of barbiturates by post-column ionization and detection by a multiplace photodiode array spectrophotometer

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(First received August 17th, 1987; revised manuscript received March 21st, 1988)

Recently, we showed that high-performance liquid chromatography (HPLC) with photodiode array detection, an electronic library of ultraviolet (UV) spectra and a computerized search algorithm can be used to identify basic and neutral psychotropic drugs quite reliably [1, 2]. Therefore, we suggested that this technique will develop into a valuable analytical tool for clinical toxicology, complementary to immunochemistry, thin-layer chromatography and gas chromatography.

The chromatographic performance of the system in the determination of acidic psychoactive drugs, in particular of barbiturates, will now be described. Furthermore, a technical modification of the system has been realized, applying a long-standing technique of UV spectroscopy [3] to the HPLC–photodiode array system: modification of the UV spectra of eluted compounds by post-column mixing of shift reagents with the eluent. In the present work this modification was confined to variation of the pH of the eluent, as pH-dependent shifts of UV spectra are well documented in toxicological standard bibliography (e.g. refs. 3 and 4). However, numerous other applications are conceivable [5], such as enhancement of selectivity and sensitivity or elucidation of molecular structures [6]. This last report is to our knowledge the only one so far that has been realized before with an HPLC–photodiode array system. But our system differs from this one by the use of a multiplace photodiode array detector equipped with two flow-cells. The shift reagent was added between them, and UV spectra of eluted compounds in the unchanged and the modified eluent were collected sequentially. Thus, reten-

0378-4347/88/$03.50

tion time and UV spectra in acidic and in basic environment, all useful criteria for identification of an unknown peak, are obtained in a single chromatographic run.

The applicability of the system will be illustrated by means of a case of acute overdosage. The intricacy of this case displays a common problem of hospital toxicology, when the analytical screening of the body fluids reveals a mixture of compounds. This may be due to (1) metabolites of the toxicant, (2) mixed poisoning that is increasing over single-substance poisoning (J. Velvart, Swiss Toxicological Information Center, personal communication) and (3) drugs that have been applied to the poisoning victim mainly for life-threatening conditions before toxicological sampling is performed: these drugs may interfere with the analytical efficacy of the system.

EXPERIMENTAL

We used essentially the same equipment and reagents as in our previous work on the analysis of basic and neutral drugs [1, 2]. In brief, a U6K injector (Waters Assoc., Millford, MA, U.S.A.) was connected to a 250 mm × 4.6 mm I.D. column filled with deactivated ODS (LC18DB Supelco, Bellefonte, PA, U.S.A.). The HPLC pumps were purchased from Waters (M590) and from Kontron (Model 414, Zurich, Switzerland). The ionic strength of the mobile phase was less than that described in our previous report [1], in order to prevent salt precipitation after addition of the shift reagent: 520 g of 0.05 m*M* potassium phosphate buffer with 1.43 μmol triethylamine (pH 2.6) was weighed into a graduated cylinder, 80 ml of methanol were added, and the volume was made up to 1 l with acetonitrile. The solution was filtered (Millipore, Bedford, MA, U.S.A.) under controlled reduced pressure (100 mbar) (partial vacuum controller, Meltec, Dübendorf, Switzerland). The flow-rate of the mobile phase was 0.5 ml/min for 10 min, 1 ml/min for a further 10 min and 2 ml/min for the last 5 min of a run. The detector, an HP 8450A photodiode array spectrophotometer (Hewlett Packard, Palo Alto, CA, U.S.A.) was equipped with two flow-cells (No. 178.8, Hellma, Munich, F.R.G.). They were connected by a Tee low-dead-volume connector (Supelco) and 45 cm of PTFE tubing (0.25 mm I.D.), coiled on the piston of a 1-ml plastic syringe. The pH shift reagent consisted of 0.1 *M* diammonium hydrogenphosphate, adjusted to pH 10.5 with 900 ml of ammonia and 100 ml of acetonitrile. It was supplied at a flow-rate of 0.1 ml/min.

The absorbance spectra in the unchanged eluent were recorded from 200 to 400 nm and from 220 to 400 nm in the basic environment. The truncation in the low UV region was necessary owing to high baseline noise. A microcomputer (HP 85B) equipped with a hard disk (HP 9153) and a 7440 plotter was used to record the data.

A variable-wavelength UV detector (Technicon Instruments, Tarrytown, NY, U.S.A.) was installed in series with the photodiode array detector and connected to an integrator (SP 4270, Spectra Physics, San Jose, CA, U.S.A.) enabling quantification.

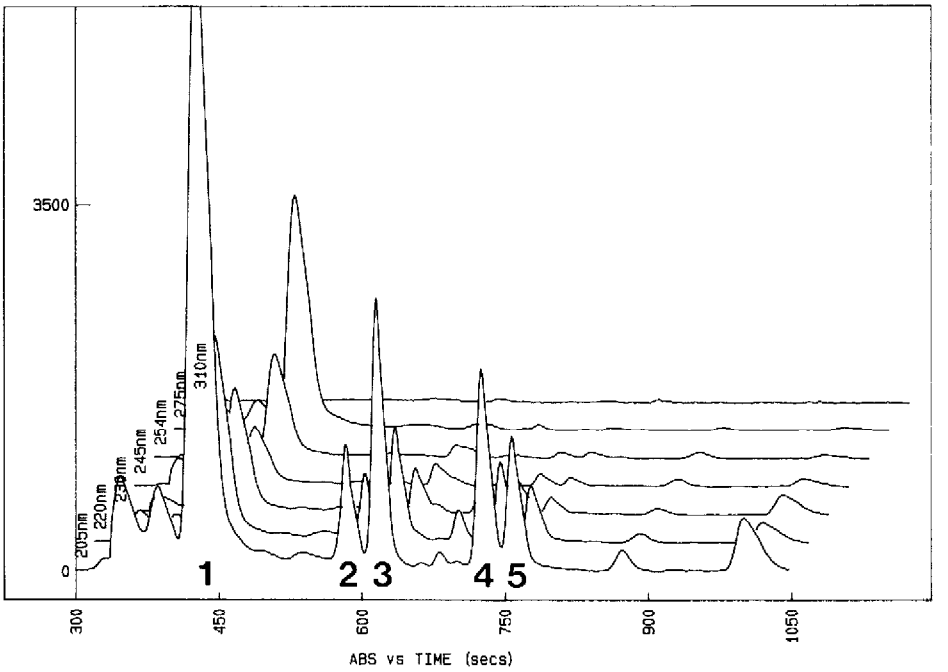

Fig. 1. Pseudo-three-dimensional multi-wavelength chromatogram of a blank serum spiked with 1 μg/ml each of phenobarbital (2), allobarbital (3), diphenylhydantoin (4) and heptabarbital (5). Peak 1 consists mainly of caffeine. The chromatographic conditions are described in Experimental.

Sample preparation

To screen for acidic xenobiotics in serum a 1-ml sample was adjusted to pH 4 with 1 *M* phosphate buffer and extracted with 5.5 ml of hexane–dichloromethane–ethanol (124:172:25, w/w/w). The organic phase was evaporated, the residue was dissolved in 100 μl of mobile phase, and 50 μl of the resulting solution were injected. The only chromatographic peaks shown by drug-free serum from healthy volunteers processed in this manner were caffeine and other methylxanthines that elute in the front region of the chromatogram (Fig. 1, peak 1 and two smaller peaks preceding it).

RESULTS AND DISCUSSION

Screening of barbiturates in body fluids by HPLC has been proposed for clinical and forensic toxicology [4]. The use of a photodiode array detector enables the on-line registration of UV spectra of eluted compounds, but decreases the selectivity of detection and therefore requires a sample preparation with low levels of interfering endogenous compounds. The extraction method developed for acidic drugs revealed only minor chromatographic peaks in relation to drugs spiked at a concentration of 1 μg/ml (Fig. 1), which is below the toxic range of barbiturates. The recoveries of four acidic drugs (phenobarbital, allobarbital, diphenylhydantoin and heptabarbital) at concentrations of 1 μg/ml ranged between 85

TABLE I

RETENTION VOLUMES AND CHROMATOGRAPHIC RESOLUTION OF BARBITURATES

Retention volumes have been calculated according to the equation:
retention volume = flow-rate × retention time (in μl). R factors have been calculated according to: $R = \frac{\text{retention volume}_2 - \text{retention volume}_1}{(\text{width}_1 + \text{width}_2)} \times 2$ where retention volume $_1$ and width$_1$ (at baseline) are the parameters of the first eluted compound, and retention volume$_2$ and width$_2$ are those of the second compound of two adjacent peaks.

Substance	Retention volume	R factor
Proxibarbal	716	
		1.31
Barbital	1125	
		2.28
Allobarbital	2034	
		0.56
Aprobarbital	2258	
		0.28
Phenobarbital	2375	
		0.42
Brallobarbital	2558	
		1.54
Butobarbital	3200	
		0 35
Cyclobarbital	3358	
		0 22
Butalbital	3458	
		2 27
Pentobarbital	4567	
		0 19
Heptabarbital	4675	
		0.08
Vinylbital	4717	
		0.19
Amobarbital	4816	
		0.73
Hexobarbital	5233	
		0 91
Methylbarbital	5767	
		1.06
Secobarbital	6375	
		5.20
Thiopental	9350	
		5.65
Febarbamat	13350	

and 91%. Standard curves of phenobarbital and allobarbital, with barbital (10 μg) as internal standard, were linear between 5 and 50 μg/ml.

Table I gives the retention volumes (corrected for dead volume) and the resolution of two adjacent peaks by R factors [7]. They reveal that most of the ana-

lysed barbiturates can be distinguished from each other, as proved experimentally by cochromatography of appropriate standards. The retention times (or analogous parameters such as capacity factors or relative retention times) have been used for the identification of barbiturates: if an appropriate standardization is performed, even inter-laboratory variations are low and therefore these terms are sufficient for identification [8]. In order to determine the variations of the parameter analogous to the retention time in our system, the retention volume of proxibarbal was measured 21 times over two months. Its mean value ($\pm$S.D.), corrected for dead volume (determined by sodium nitrate), was 715.5 ± 16.7 μl, corresponding to a coefficient of variation of 2.3%. This low variation indicates that the retention volume may serve as a useful criterion for drug identification, in addition to the on-line UV spectra. Its application within an electronic search algorithm for a library of UV spectra has been discussed in our previous paper [1].

The ability of this system to separate individual barbiturates is slightly lower than that of two published methods that use a basic mobile phase [9, 10], but it enables the use of the same instrumentation, including column and eluent, as for our assay of basic and neutral drugs [1]. Thus, the basic/neutral and acidic fractions of a body fluid extract can be analysed without time delay by reequilibration of the instrumentation, a feature useful in emergency cases.

In hospital toxicology, one is often faced with the fact that the analytical specimen contains a mixture of drugs, as mentioned above. This may be of toxicological significance, if a combined poisoning is present or the mixture consists of metabolites of the toxicant. But often one has to deal with therapeutically applied medication, which potentially interferes with the chromatographic analysis of the toxicant(s) of interest. If such a drug mixture is present in the sample, the different peaks may elute in an overlapping sequence that results in distortion of their UV spectra. A number of mathematical tools that make use of the inherent capabilities of multi-wavelength chromatography have been developed to deal with this problem. By this means the analyst can judge peak purity [11], resolve underlying pure spectra and compute concentration–time curves for overlapping peaks [12–16]. The latter techniques require considerable computing power and therefore are not readily applicable to hospital toxicology. Our practical approach to this problem was to restrict our analysis to UV spectra gathered at the apices of the chromatographic peaks. A suitable ratio of the main component to the interfering one results in most instances.

In addition, our system can register a second on-line UV spectrum subsequently to the addition of the basic reagent, which may produce a shifting of the UV spectra by ionization or deprotonization of analytes. An induced or missing change in the shape of an unknown UV spectrum can be compared with the standard bibliography of toxicology [3, 4] and thereby yield increased certainty in identification. As the organic modifier contained in the mobile phase may influence the spectra to some extent (A.C. Moffat, personal communication), confirmation of the results by the analysis of pure standards is advisable. An electronic library of UV spectra recorded under standard conditions, combined with a search algorithm, is suitable in this context [1, 17–19]. The additional information pro-

vided by the shift reagent proved useful for the identifications of basic and neutral drugs (unpublished results), but was mainly evident for the analysis of barbiturates. The on-line UV spectrum and its characteristic shift principally serve to identify the eluted compound as a barbiturate. The retention volume is sufficient to identify the individual barbiturate in most instances. As an additional feature some variation in the low UV region of individual acidic spectra may be observed.

An illustration of the method described is given by a chromatogram derived from a plasma extract of a poisoning victim, who was brought into hospital with delay and required immediate therapy for seizures and for pulmonary infection by diazepam and diphenylhydantoin and by sulphamethoxazol/trimethoprim, respectively. The immunochemical screening revealed a urine positive for barbiturates, and on the following day a plasma sample was obtained for confirmation of this finding and differentiation of the barbiturates by HPLC. The chromatographic analysis is given in Fig. 2. Four pairs of spectra collected during the run and marked on Fig. 2 are displayed as diagrams 1–4 in Fig. 3. Identical digits correspond to one another. In addition to the front peak (mostly caffeine), five distinctive components were found in the chromatogram. The first peak corresponds to sulphamethoxazole, the sulphonamide component of the antibacterial agent. The second and fourth are barbiturates: the second brallobarbital, the fourth secobarbital. The third component is in a much higher concentration available and has a spectrum similar to barbiturates in acidic medium but no basic shift, and corresponds to diphenylhydantoin. The last component (spec-

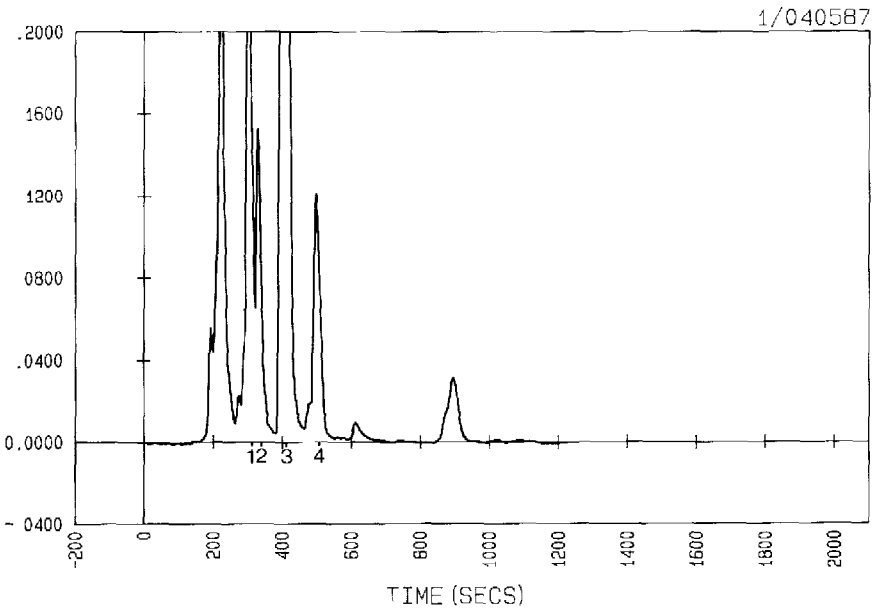

Fig. 2. Chromatogram of a serum extract, registered at a flow-rate of 1 ml/min. It was obtained from a patient who had ingested an overdose of Vesperax® (brallobarbital, secobarbital and hydroxyzine). The patient was treated for epileptic seizures by diazepam and diphenylhydantoin and for pulmonary infection by Bactrim® (trimethoprim, sulphamethoxazole) prior to this blood sample being withdrawn. Numbers 1–4 correspond to the spectra depicted in Fig. 3.

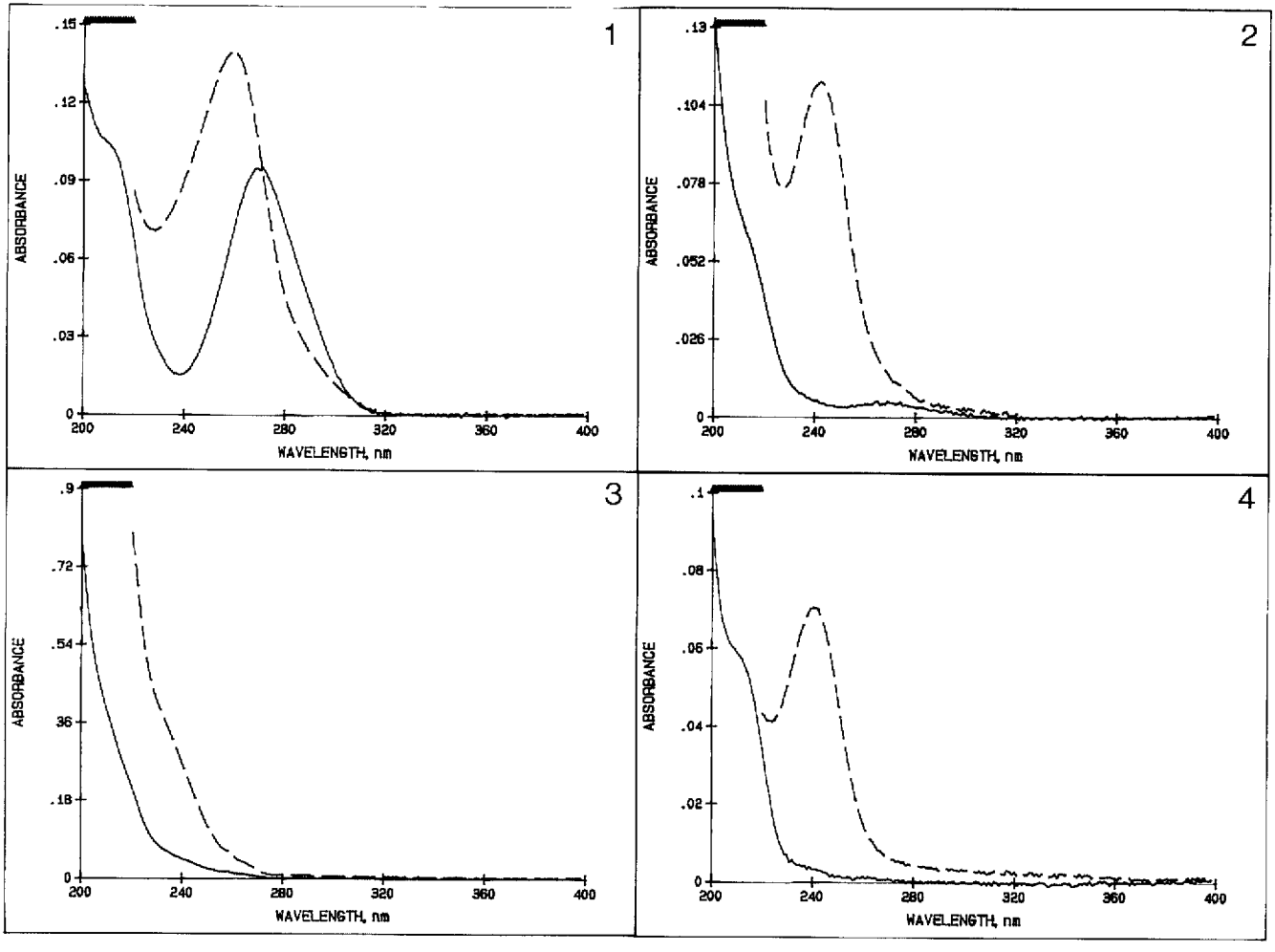

Fig. 3. Some UV spectra, gathered on-line, of the chromatogram in Fig. 2. Those collected in the acidic eluent are displayed by a solid line. Those collected in basic mobile phase after the addition of the shift reagent are depicted by a broken line. (1) Sulphamethoxazole; (2) brallobarbital; (3) diphenylhydantoin; (4) secobarbital.

trum not shown) exhibits an absorbance maximum at 230 nm, a shoulder at 250 nm and a second maximum at 311 nm without changes between acidic and basic registration. It matches diazepam by retention volume and UV spectra.

It was later confirmed that the patient had ingested an overdose of Vesparax®, a formulation that contains two barbiturates, brallobarbital and secobarbital, and also hydroxyzine, an antihistaminic. This last component was not found, most probably owing to its short biological half-life of 3–4 h and its high volume of distribution, both leading to a plasma concentration below the detection limit of our system.

CONCLUSIONS

HPLC has a broad detection and identification capability [21] within an analytical screening programme for toxicants in body fluids [22], especially if used with a photodiode array detector [1] and a post-column ionization step. Its usefulness and practicality have been tested by the identification of barbiturates.

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