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Combined pH/organic solvent gradient HPLC in analysis of forensic material

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Abstract

A combined pH/organic solvent linear gradient mode in high performance liquid chromatography (HPLC) is presented as a new approach to determination of low concentrations of ionogenic analytes in biological material. The approach consists in simultaneous development of linear gradients of pH and organic modifier in the mobile phase. Advantages of the method are illustrated in postmortem analysis of opipramol in material from suicide victims. Very narrow peaks without tailing were obtained and several times lower limits of analyte quantitation were achieved using ultraviolet detection as compared to a standard isocratic method. The double gradient HPLC method seems to be especially valuable in case of ionogenic analytes dispersed in complex biological matrices. That is due to a high selectivity of the double gradient method and the lack of peak tailing, which is commonly observed for basic analytes chromatographed at isocratic conditions.
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1. Introduction

The influence of pH on the retention of analytes in reversed phase high-performance liquid chromatography (RP HPLC) has extensively been studied [1–5]. The retention of an ionized form of analyte is several times lower than retention of nonionized form. The general dependence of retention factor, *k*, on pH is similar to pH titration curve. The effect of pH on retention can be exploited in the pH gradient mode of elution. Recently, we introduced pH gradient RP HPLC and the combined pH/organic solvent gradient RP HPLC [6–9]. We also succeeded in describing the approach in strict theoretical terms [8–10].

The pH gradient RP HPLC methods proposed consist in programmed change of eluting power of mobile phase during chromatographic run with respect to the acid/base analytes separated. In case of combined pH/organic solvent gradient, the increase of eluting power is realized by linear changes of pH, accompanied with a simultaneous linear increase of organic modifier content in the mobile phase. During the chromatographic run pH increases in case of acidic analytes and decreases in case of basic analytes.

The main advantage of the combined pH/organic solvent gradient mode is compression of the peaks [11]. It is assumed that during the elution, at any site in the column, the analyte molecules passing through it earlier are exposed to a weaker eluent than the molecules that pass through it later. A stronger eluent (lower pH, higher methanol content) pushes base analytes faster than a weaker eluent preceding it (higher pH, lower methanol content). Thus, the "tail" is permanently being pushed back into main peak and peak widening is reduced. Maximum peak compression can be expected if retention of analyte occurs after eluent pH passed the range at which analyte retention is changing due to its changing dissociation degree. That happens at pH region around ${}^{s}_{s}pK_a \pm 1.5$. In such

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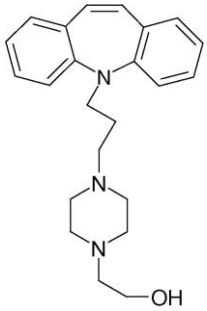

Fig. 1. Structure of opipramol.

a case, during the simultaneous pH/organic solvent gradient run an increase of methanol content causes a decrease of retention of the analyte. However, near the moment of elution the analyte is still under organic solvent gradient conditions and is subjected to quite a strong retention. Then, the changing pH rapidly decreases its retention. From that moment the analyte is no longer significantly retained and a narrow peak is observed.

Retention in the pH/organic solvent gradient mode is determined by lipophilicity of both the ionized and the nonionized form of the analyte as well as by its pK_a. That should provide a high selectivity of the method. In other words, separation conditions may be adjusted in such way that only the selected analytes are eluted at actual pH gradient mode. The remaining analytes, including those originating from matrix, are eluted when organic solvent gradient alone is operating. Therefore, for those later eluting analytes less pronounced peak compression is observed than that obtained for the selected analytes of interest.

To test the usefulness of the combined pH/organic solvent gradient mode of RP HPLC in analysis of biological samples, a basic psychotropic drug, opipramol (Fig. 1), was analyzed in biological material from a suicide victims. Standard HPLC procedures for determination of that drug in forensic medicine are troublesome and of disputable reliability. Samples of blood, stomach content, urine, brain, kidney and liver were taken postmortem from victims of fatal opipramol overdosing. The samples to be analyzed are characterized by a very complex matrix, which sometimes makes standard isocratic RP HPLC analysis practically impossible due to many interfering components.

2. Experimental

2.1. Equipment

The HPLC system applied was Merck–Hitachi LaChrome (Darmstadt, Germany–San Jose, CA, USA) of the dwell volume, V_d, of 1.4 ml, equipped with a diode array detector, autosampler and thermostat. Chromatographic data were collected using D-7000 HPLC System Manager, Version 3.1 (Merck–Hitachi). The column was XTerra MS C-18, 150 mm × 4.6 mm i.d. (Waters Corporation, Milford, MA, USA), packed with octadecylbonded silica of particle size of 5 μm. Mobile phases contained methanol as the organic modifier. A 1% urea was a dead volume marker, V_0. The estimated V_0 was 1.72 ml. Flow rate was 1 ml/min.

2.2. Mobile phase

Mobile phase contained methanol as the organic modifier (solvent B). Buffers of fixed pH formed the aqueous component of the eluent. Essential aqueous solvent contained citric acid in concentration of 0.02 M. Buffers of $^{w}_{w}$pH 3.0 (buffer I) and $^{w}_{w}$pH 6.0 (buffer II) were made by pH adjustment with 1 M HCl and 1 M NaOH, respectively. Buffers I and II, mixed at various proportions during the chromatographic run, provided linear change of eluent pH. The pH of the buffers was measured at 25 °C with an HI 9017 pH-meter (Hanna Instruments, Bedfordshire, UK).

2.3. Case report

Husband of the victim I.K. reported that, on the day she died, she came back home in the late evening. He saw she was sick and vomiting. After some hours he found her dead in her room. During the investigation at the death place, the police found some empty containers of the following drugs: opipramol, clometiazole and heminevrin. The husband of dead female also mentioned, that his wife had been abusing alcohol and was treated against this addiction. During the autopsy the medical examiner noted the cause of death as the asphyxia due to vomiting, but he did not exclude the death due to drug overdosage.

The body of the second victim M.K. was found in the summerhouse with the plastic bag put on his head, with no injuries on his body noted. During the investigation it was established that the victim had been overusing alcohol and used to inhale some toxic substances to achieve the state of dizziness. By the autopsy the medical examiner noted the cause of death as the asphyxia due to closing mouth and nasal ducts by the plastic bag, but because of finding some unidentified softening tablets in the stomach, the examiner also did not exclude the cause of death due to drug overdosage.

2.4. Extraction procedures

A 20 ml of blood and urine samples and 20 g of kidney, brain, liver and stomach content samples were taken postmortem according to the protocol accepted by Local Ethic Commission of the Medical University of Gdańsk. Samples were homogenized and extracted on Extrelut columns (Merck, Darmstadt, Germany–San Jose, CA, USA). Opipramol samples after alkalization with 0.1 M NaOH were extracted from basic pH with dichloromethane/isopropanole, 85/15 (v/v). The obtained extracts were evaporated to dryness and the residue was redissolved in 20 ml of ethanol. The ethanolic solutions were introduced onto the column.

3. Results and discussion

Optimization of gradient program for analysis of opipramol samples by the combined pH/organic solvent double linear gradient RP HPLC started with a measurement of retention of the analyte in two methanol gradient runs of a wide methanol concentration range, differing in gradient development time. The established pH 6.0 of the mobile phase at the beginning of gradient was high enough to suppress dissociation of opipramol (pK_a 4.5). From those two experiments, retention time of opipramol was evaluated from linear solvent strength theory (LSS) [12] for required methanol gradient program by means of DryLab software (LC Resources, Walnut Creek, CA). The predicted gradient retention time was confirmed experimentally. Next, the pH gradient was over imposed onto the organic gradient in such a way that changing pH affected retention of opipramol directly before its elution from the column. In case of opipramol, the maximal changes in retention connected with pH gradient were at the pH range of p$K_a \pm 1.5$, i.e., at the pH falling down from 6 to 3. Thus, the elution of opipramol was expected to take place at the end of the applied pH gradient (pH 3.0). Including the pH gradient one must have in mind that composition of mobile phase at column outlet is delayed for $t_d + t_0$ as compared to the composition supplied by the pump. Here, t_d is dwell time of the chromatographic system and t_0 is column dead time.

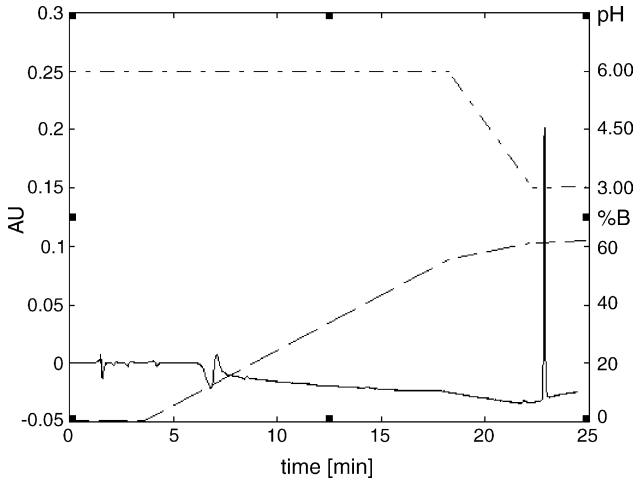

Fig. 2. Chromatogram of standard opipramol from a combined pH/organic solvent gradient (conditions, see Table 1) RP HPLC run. Dash-doted line shows changes in pH and the dashed line shows changes in methanol content at column outlet.

Table 1
Pump program for pH/organic gradient run corresponding to chromatogram obtained in Fig. 2

Time (min)	Methanol (%)	Buffer (pH 3.0) (%)	Buffer (pH 6.0) (%)
0	0	0	100
15	57	0	43
19	62	38	0
25	62	38	0

Figs. 2 and 3 show the chromatograms of opipramol samples obtained at two different gradient programs. Respective gradient programs (pump programs) are given in Tables 1 and 2. On the chromatograms (Figs. 2 and 3) also the changes in pH and methanol content at column outlet are shown: the dash-doted line shows changes in pH and the dashed line shows changes in methanol content. It can be noted that the position of the peak (when the pH gradient is de-

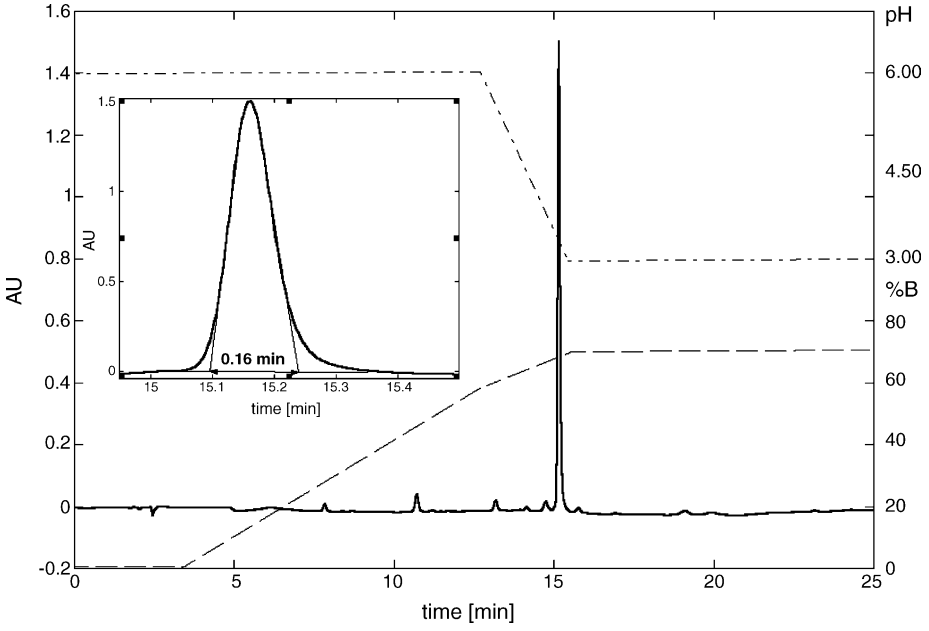

Fig. 3. Chromatogram of a brain extract sample from a combined pH/organic solvent gradient (conditions, see Table 2) RP HPLC run. A 5 μl portion of sample was applied onto the column. Dash-doted line shows changes in pH and the dashed line shows changes in methanol content at column outlet.

Table 2
Pump program for pH/organic gradient runs corresponding to chromatograms obtained in Figs. 3 and 4

Time (min)	Methanol (%)	Buffer (pH 3.0) (%)	Buffer (pH 6.0) (%)
0	0	0	100
10	60	0	40
12	70	30	0
25	70	30	0

veloped near the elution time of the analyte) depends mainly on the methanol gradient program. Generally, the steeper is the gradient or/and the higher is the content of methanol at the beginning of gradient program the faster is the elution of the analyte. Any changes in organic modifier gradient program require an appropriate adjustment of the pH gradient program, to obtain an optimal bandwidth of the peak.

The best opipramol analysis data were obtained when pH gradient ended at the time of analyte elution from the column. Then, one can get a very narrow peak. Its bandwidth might be less than 0.2 min (Fig. 2). The peak bandwidth obtained with methanol gradient alone is about 0.6 min. Hence, the peak in combined pH/organic solvent gradient RP HPLC is about three times in average higher than at the organic gradient alone conditions. It is obviously several times higher than a corresponding peak obtained at isocratic conditions.

Peak compression is of special importance when the peaks are not separated completely as illustrated in Fig. 4. After enlargement of a respective part of the chromatogram, it is evident that a narrow peak of opipramol arises on the arm of an interfering peak. In spite of that, opipramol peak can easily be identified and integrated.

Next, the optimized pH/organic double linear gradient approach for the determination of opipramol in forensic samples was tested as regards method's linearity and repeatability. The linearity was investigated at the concentration range from 0.04 to 40 ng/μl and the determined square of the correlation coefficient was $R^2 = 0.9999$. Mean retention time of opipramol was 15.11 ± 0.03 min ($n = 22$). Repeatability of the retention time readings, expressed by relative standard deviation (R.S.D.%), was 0.18%.

In the kidney, urine and liver samples analyzed the method applied was unable to detect measurable quantities of opripramol. The concentration found in brain of suicide I.K. was 820 μg/ml; in stomach content it amounted to 4.69 mg/ml in case M.K. and 5.6 mg/ml in case I.K. The concentration of opipramol determined in blood of the suicide I.K. was 3.34 μg/ml.

4. Conclusions

A simultaneous pH/organic solvent double linear gradient mode is a new RP HPLC separation method that extends analytical versatility of the chromatographic techniques. It can be freely accomplished using regular HPLC equipment. The approach is characterized by very narrow analyte peaks and hence, by increased detection sensitivity. The method can be recommended for analysis of samples of complex matrix and/or in situations when small amounts of analyte have to be assayed. However, when using the technique one must take into consideration possible problems arising from the complex nature of the combined pH/organic solvent gradient like baseline instability, need of equilibration of the column after each run and difficulties in optimization of separation condi-

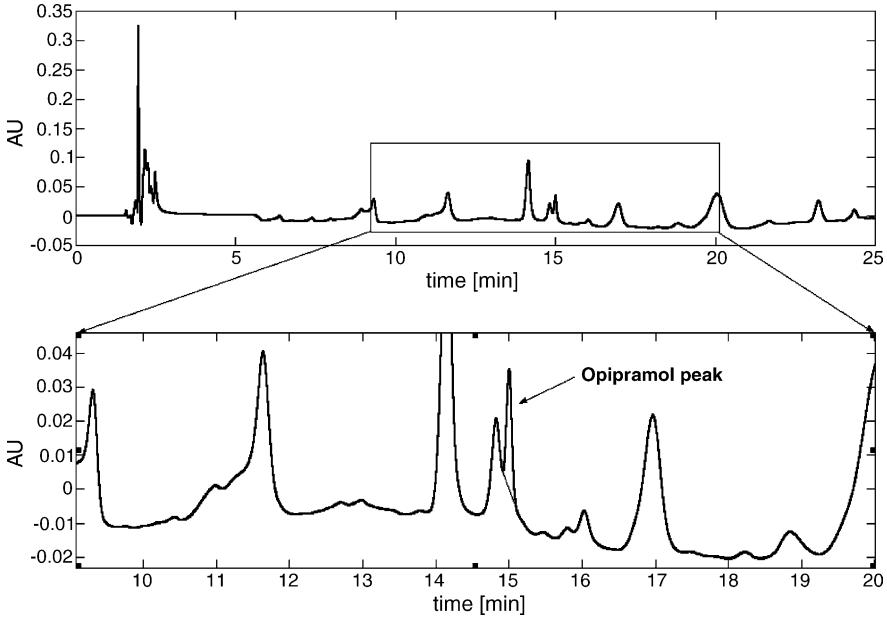

Fig. 4. Chromatogram of brain extract sample from a combined pH/organic solvent gradient (conditions, see Table 2) RP HPLC run. A 50 μl portion of sample was applied onto the column.

tions for two or more analytes simultaneously. The method has no advantage for neutral analytes. However, in case of ionogenic analytes, like majority of the existing drugs, the newly developed method might be a useful alternative to the standard HPLC approaches.

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