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Simultaneous separation and quantitation of four antiepileptic drugs—a study with potential for use in patient drug level monitoring

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Abstract

The purpose of this study was to illustrate the applicability of high-performance liquid chromatography (HPLC) coupled with evaporative light scattering detector (ELSD) in simultaneously separating and quantitating four commonly used antiepileptic drugs (AEDs). A mixture of the four AEDs were separated using a C8 column using volatile mobile phases and were detected using ELSD. Optimal instrumental conditions were obtained by assessing the effect of various critical experimental parameters such as evaporator tube temperature, carrier gas flow rate, photomultiplier gain on separation efficiency, accuracy, reproducibility and sensitivity of measurement on all four AEDs. A novel, rapid, accurate, sensitive, reproducible and robust HPLC–ELSD method for simultaneous separation and quantitation of four commonly used AEDs was developed. The physical basis of the results obtained as a consequence of varying several critical experimental parameters has been explained. This study illustrates the potential for use of HPLC–ELSD in drug level monitoring of patients undergoing mono- or polytherapy for epilepsy.

Keywords: Antiepileptic drugs; Antiepileptic drug monitoring; Evaporative light scattering detection; Sodium valproate; Polytherapy

1. Introduction

Patients suffering from refractory epilepsy, multiple seizure types and those infected with human immunodeficiency virus (HIV) are often co-medicated with multiple antiepileptic drugs [1–8]. A few of the common antiepileptic drugs administered, whose chemical structures are given in Fig. 1, are sodium valproate (VPA-Na (**1**), marketed under the brand name Depacon® by Abbot Laboratories), primidone (PRM (**2**), marketed under brand name Mysoline® by Elan), carbamazepine (CBZ (**3**), marketed under the brand name Carbatrol® by Shire Richwood

Abbreviations: HPLC, high-performance liquid chromatography; ELSD, evaporative light scattering detection (detector); HIV, human immunodeficiency virus; VPA-Na, sodium valproate; PRM, primidone; CBZ, carbamazepine; UV, ultravoilet; RI, refractive index; CE, capillary electrophoresis; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; C8, octyl silica; ml, milliliter; μg, microgram; μl, microliter; DL, detection limit; QL, quantitation limit; R.S.D., relative standard deviation; °C, degree centigrade; PEG, poly ethylene glycol; M, molar; AED, antiepileptic drug

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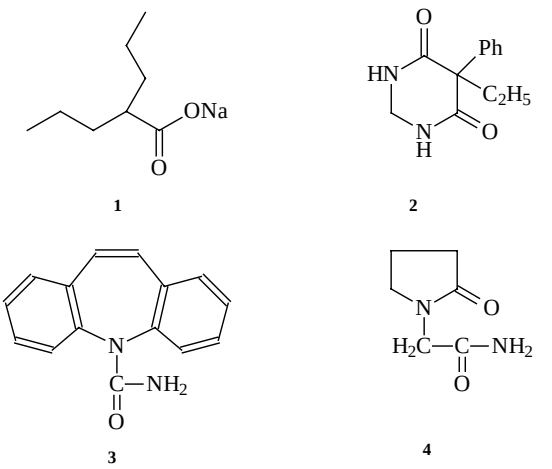

Fig. 1. Chemical structures of antiepileptic drugs: (1) VPA-Na, (2) PRM, (3) CBZ and (4) piracetam.

and Tegretol® by Novartis) and piracetam (**4**, marketed in the UK for treatment of myoclonus). During recent years, treatment of epilepsy using VPA-Na has gained considerable momentum. However, when VPA is co-administered with drugs such as PRM or CBZ, drugs that can elevate the levels of glucoronyl transferase, the concentration of VPA-Na levels in the patient is dramatically reduced. Conversely, the levels of CBZ and PRM in the patients' body are increased, to different extents, upon co-administration with VPA-Na [9]. Pharmacokinetic interactions also exist between the other aforementioned antiepileptic drugs and represent a major complication of epilepsy treatment with polytherapy. Since the therapeutic index of these drugs is normally narrow, especially in pediatric patients, rapid, accurate and simultaneous pharmacokinetic monitoring is required to achieve effective treatment.

These four antiepileptic drugs studied exhibit wide variances or exclusivity in solubility characteristics and stark differences or slight variability in absorption characteristics. While it may be possible to simultaneously analyze piracetam, PRM and CBZ using high-performance liquid chromatography (HPLC) with multiwavelength UV detection, it is not possible to analyze VPA-Na using UV detection owing to its lack of a chromophore. In fact, published literature to-date analyzes VPA either by CE [10], GC with pre-column derivatization [11], GC–MS [12], fluorometry with derivatization [13], HPLC–UV with pre-column derivatization [14] or HPLC with fluorescence detection after derivatization [15]. Derivatization techniques are often undesirable due to a variety of problems such as lack of reproducibility and robustness.

Evaporative light scattering detection (ELSD) has been used increasingly in recent years for compounds that have no absorption in the UV spectrum or those which do only at the UV wavelength extremities where sensitivity can be severely compromised [16,17]. In principle, this technique can detect any solute that is less volatile than the mobile phase itself. In HPLC–ELSD, the eluent from the column is nebulized into small droplets by means of a controlled gas stream, after which the mobile phase is evaporated in the evaporation chamber with controlled heat. Finally, the analyte molecules are detected based on the light scattered when they pass through a light stream. The intensity of light scattered depends on a variety of factors including size, shape and surface properties of the particle. ELSD is advantageous compared to refractive index (RI) detection because of increased sensitivity, compatibility with gradient elution and non-susceptibility to ambient temperature variations during analysis. ELSD has been used to detect and quantitate various analytes from biological fluids [18–21], combinatorial libraries [22,23], drug delivery vehicles [24,25], non-ionic surfactants [26,27] and hydrophilic carriers [28], thus demonstrating its versatility as an important tool in the area of pharmacognosy.

In this paper the author discusses the development and results of a novel, rapid, accurate, reproducible, sensitive and robust method utilizing HPLC–ELSD for the simultaneous separation and quantitation of four antiepileptic drugs.

2. Materials and methods

2.1. Chemicals and reagents

Piracetam (2-oxo-1-pyrrolidineacetamide (**4**), Fig. 1), VPA-Na (2-propylpentanoic acid, sodium salt (**1**), Fig. 1), PRM (2-desoxyphenobarbital (**2**), Fig. 1) and CBZ (5H-dibenz[*b*, *f*]azepine-5-carboxamide (**3**), Fig. 1) were purchased from Sigma Chemical Co., St.

Louis, MO, USA (Cat. No. P-5295, P-4543, P-7295 and C-4024, respectively) and were used as received. Ammonium acetate was purchased from Mallinckrodt, USA (Cat. No. 3272). Isopropyl alcohol (2-propanol) was obtained from VWR scientific products, West Chester, PA, USA (Cat. No. VW5540). Ethanol was obtained from Pharmaco products Inc. Brookfield, CT, USA (Cat. No: DSP-CT-18). Distilled water was purified using Millipore water purification systems (Cat. No. ZMQS6V00Y) utilizing Quantum™ EX cartridge (Cat. No. QTUM000EX) and Q-Gard™ 2 purification pack.

2.2. Chromatographic system

A Waters® (Milford, MA, USA) liquid chromatographic system, separation module 2690, consisting of an auto injector and pump (configured to continuous vacuum) was used. Data were acquired and processed using Millenium^{32} (Version 3.20) software from Waters®. The column used was a Hibar pre-packed column RT 250-4, Lichrosorb® RP-8 from Merck KgaA, Darmstadt, Germany. The column dimensions were 250 mm × 4 mm with a 5 μm stationary phase particle size. An external column heater, Metatherm™ supplied by Metachem®, Torrance, CA was used to maintain the column temperature at 25 °C during analysis. The flow rate was maintained at 0.5 ml/min and the injection volume was 20 μl.

2.3. Elution conditions

Gradient elution conditions were used to achieve the desired separation between the four drugs in a timely manner. The solvents used were (A) 0.01 M ammonium acetate (B) ethanol and (C) isopropyl alcohol. Gradient conditions are given in Table 1. No pH adjustments were performed.

2.4. Detector settings

The detection was achieved using a Sedex® 55 (Sedere, France) evaporative light scattering detector (ELSD). The evaporator tube temperature was maintained at 30 °C. The carrier gas (purified nitrogen) flow rate was set at 1.5 bar and the photomultiplier gain was set at 9.

Table 1
Gradient conditions used for analysis of the four AEDs under study

Step	Time	Flow (ml)	A (%)	B (%)	C (%)	Curve
1	0	0.5	60	25	15	
2	8	0.5	60	25	15	6[a]
3	12	0.5	30	70	0	2[b]
4	14	0.5	60	25	15	2[b]

(A) 0.01 M ammonium acetate, (B) ethanol, (C) isopropyl alcohol.
[a] Represents linear progression of gradient.
[b] Represents convex progression of gradient.

2.5. Standard preparation

Stock solutions of each of the four antiepileptic drugs were prepared by sonicating and dissolving accurately weighed portions of the drugs in ethanol. Aliquote of the stock solutions were then appropriately ratioed and mixed in a volumetric flask and diluted to volume with ethanol to yield column loads of 3.7760 μg of piracetam, 8.8688 μg of VPA-Na, 3.267 μg of PRM and 7.8796 μg of CBZ for a 20 μl injection volume.

3. Results and discussion

The chromatographic method was developed to provide optimal results primarily for VPA-Na, since it is a broad-spectrum antiepileptic, non-chromophoric and would thus provide maximum applicability for clinical researchers, pharmacokineticists, pharmaceutical scientists and the like. Separation and quantitation of piracetam, PRM and CBZ were nevertheless important owing to their widespread use in mono- and poly-antiepileptic therapy. Acceptable separation, reproducibility, accuracy and sensitivity were obtained using a C8 column as the stationary phase. Due to divergent solubility characteristics of these drugs, the choice of different solvents as mobile phases was limited. The elution, chromatographic and detector conditions given above represent the best conditions for a reproducible, accurate, sensitive, robust and validatable method for simultaneously separating and quantitating these four drugs.

A gradient elution system comprising of 0.01 M ammonium acetate, ethanol and isopropyl alcohol provided a well-resolved chromatogram with retention times of 4.7, 5.7, 7.2 and 10.4 min for piracetam,

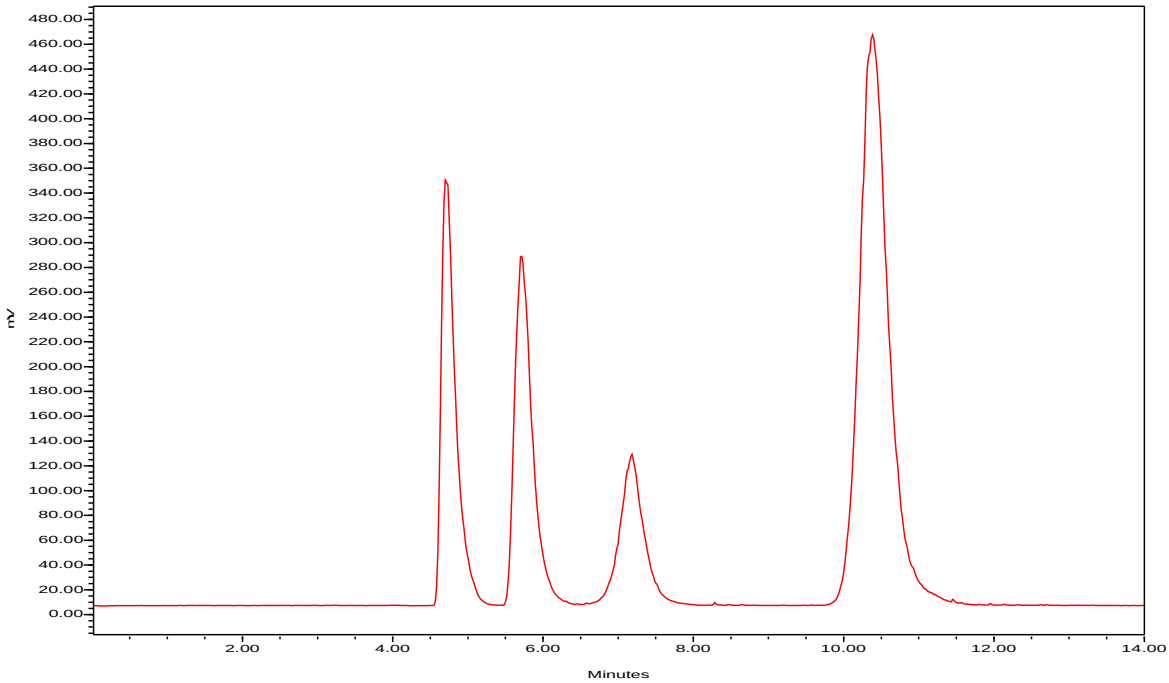

Fig. 2. A typical chromatogram of the standard preparation. Piracetam (Rt = 4.7), VPA-Na (Rt = 5.7), PRM (Rt = 7.2) and CBZ (Rt = 10.4).

VPA-Na, PRM and CBZ, respectively. A typical chromatogram of the standard preparation is shown in Fig. 2. It has generally been observed that, unlike UV detection, linear calibrations of standards are uncommon in ELS detection. Typically, the detector response, as measured by peak area, varies exponentially with the mass of the analyte. Moreover, this behavior can be mathematically expressed in logarithmic form as shown in Eq. (1):

$$\log(x_i) = \log(a) + b\log(m_i) \quad (1)$$

where the detector response x of a particular component i is related to the mass m of the same component i through constants a and b. In a plot of $\log(x_i)$ as a function of $\log(m_i)$, b corresponds to the slope while $\log(a)$ corresponds to the intercept of the regression line. A log(peak area) − log(mass) plot for VPA-Na is shown in Fig. 3. Each data point represents the average area obtained from 10 replicate injections. Acceptable correlation ($R^2 = 0.9971$) has been obtained, in these experiments. The R^2 values as well as constants a and b for piracetam, VPA-Na, PRM and CBZ, similarly obtained as mentioned above are given in Table 2. Satisfactory linearity of detector response has been simultaneously obtained for all four analytes. Repeatability and intermediate precision were measured by making replicate injections ($n = 10$) of the standard preparation. The percent R.S.D. for all the analytes was always under 2.5% and the pooled precision was 1.6%. Intra-day accuracy was established by generating a single point calibration curve and by determining the concentration of freshly prepared control based on the standard curve. The percent deviation between theoretical and calculated concentrations of the control was used as a measure of intra-day accuracy. The deviations were found to be +0.6, +0.8, −2.2 and −1.0% for piracetam, VPA-Na, PRM and

Table 2
a, b and R^2 values for the fours AEDs under study

Analyte	a	b	R^2 values
Piracetam	5.7685	1.6053	0.9986
VPA-Na	5.0466	1.7463	0.9971
PRM	5.7169	1.4118	0.9951
CBZ	5.7835	1.4836	0.9954

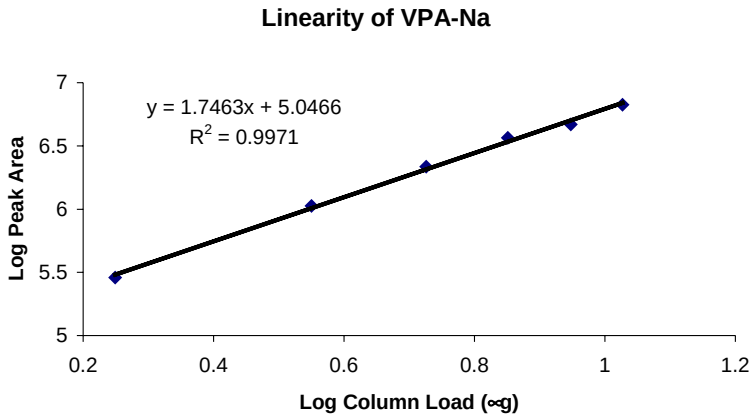

Fig. 3. A log(area) – log(mass) plot demonstrating linearity of detector response for VPA-Na.

CBZ, respectively. Inter-day accuracy was assessed in the same way as above, except that the standards were prepared fresh on the fourth and fifth day, and the concentrations of the control prepared on the first day was determined based on the curve generated by the freshly prepared standards. The percent deviation for piracetam, VPA-Na and CBZ were all found to be acceptable ($<\pm 5.0\%$) through the fifth day while the deviation for primidone was found to be $+5.8\%$ and $+17.7\%$ for the fourth and fifth day, respectively. The sensitivity of this method was assessed by calculating the detection limit (DL) and quantitation limit (QL) for all four analytes. DL was calculated according to the formula DL = 3.3(S.D./*S*), where S.D. is the standard deviation of the response ($n = 6$) and S is the slope of the calibration curve at levels approaching the DL. Similarly, QL was calculated according to the formula QL = 10(S.D./*S*), where S.D. is the standard deviation of the response ($n = 6$) and S is the slope of the calibration curve at levels approaching the QL. The DL and QL values for all four analytes, determined as described above, are given in Table 3. From these results in addition to high precision and good linearity, it is clear that this method is sensitive as well.

Table 3
Calculated DL and QL values for the four AEDs under study

Analyte	DL (μg)	QL (μg)
Piracetam	0.01	0.09
VPA-Na	0.10	0.51
PRM	0.04	0.08
CBZ	0.03	0.10

The robustness of this method was investigated by probing the effect of various chromatographic and detector parameters on individual and overall precision, peak sensitivity, separation and peak efficiency. The various parameters that were investigated were (a) evaporator tube temperature, (b) carrier gas flow rate, (c) buffer strength, (d) mobile phase flow rate, (e) photomultiplier gain, (f) analyte linearity and (g) column temperature. Each of these parameters had either a subtle but significant, or drastic effect on sensitivity or separation efficiency. All data points in the following figures represent average of 10 replicate injections.

3.1. Effect of evaporator tube temperature

The evaporator tube temperature was varied from 30 to 80 °C while maintaining all the other experimental parameters constant and data were collected pertaining to precision, response, separation and peak efficiency. A plot of the peak area as a function of evaporator tube temperature is given in Fig. 4. In order to at least qualitatively understand the effect of the various experimental critical parameters, one has to delve into the theories of nebulization (aerosol formation) and angular light scattering [29–31]. The intensity of the scattered light proportionally depends on, amongst other factors, the size of the particle in the drift tube that passes through the detector cell, which in turn depends on the size of the aerosol formed during the nebulization process. The average droplet diameter

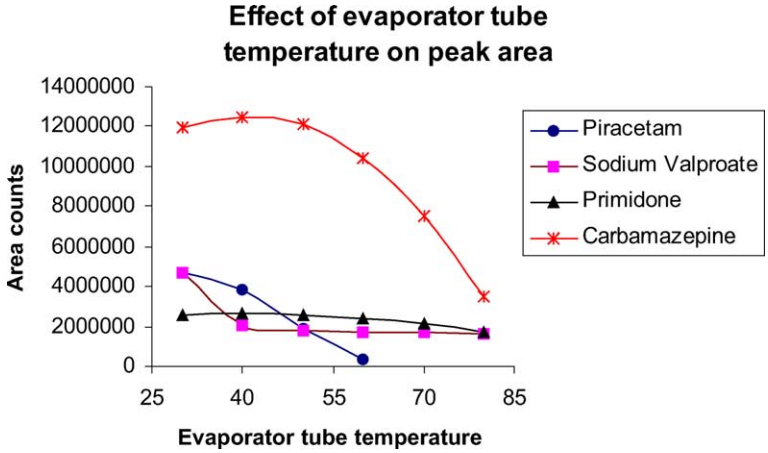

Fig. 4. Plot of peak area as a function of evaporator tube temperature for four AEDs.

(D_0) of the aerosol, formed as a result of nebulization, can be calculated using the empirical equation developed by Nukiyama and Tanasawa [32–34].

$$D_0 = \frac{585\sqrt{\sigma}}{(V_g - V_l)\sqrt{\rho}} + 597\left(\frac{\mu}{\sqrt{\sigma\rho}}\right)^{0.45}\left(\frac{1000Q_l}{Q_g}\right)^{1.5} \quad (2)$$

where D_0 is the average droplet diameter of the aerosol (μm), σ the liquid surface tension of the solution (dyne/cm), ρ the density of the solution (g/ml), μ the viscosity of the solution (poise), $V_g - V_l$ the difference in the gas and liquid velocities in the nebulizer (m/s), Q_l/Q_g is the ratio of the liquid to gas volumetric flow rates (l/min).

After the eluent from the column is nebulized, the aerosols enter the drift tube wherein the solvent in the aerosol is evaporated to produce particles of pure solute. The time, t_d, for the solvent to be completely vaporized can be calculated from the equation proposed by Charlesworth [31].

$$t_d = \frac{2\,\Delta H_v \rho D^2}{MK_f\,\Delta T} \quad (3)$$

where t_d is the time required for the solvent of the droplet to be completely vaporized, ΔH_v the latent heat of vaporization of the solvent, ρ the density of the solution, D the initial droplet diameter, M the molecular weight of the solvent, K_f the thermal conductivity of the gas film surrounding the solvent, ΔT is the difference between the air temperature and the surface temperature of the droplet.

Eq. (3) is also applicable to the evaporation of the solute (particle without the solvent), if the evaporator tube temperature is too high. Upon evaporation of the solvent from the droplet resulting in the formation of the solute particle, the particle enters the detector cell. The diameter of the particle entering the detector cell is related to the initial droplet diameter D_0 by the equation:

$$d = D_0\left(\frac{c}{\rho_a}\right)^{1/3} \quad (4)$$

where D_0 is the initial droplet diameter at the nebulizer outlet, C the concentration of the solute in the original droplet, ρ_a is the density of the analyte.

As evidenced from Fig. 4, higher temperatures lead to a decrease in peak areas of all analytes. This can qualitatively be explained by using Eq. (3). Higher temperatures result in larger ΔT, which in turn leads to shorter t_d. Shorter t_d result in smaller particles entering the detector cell which results in smaller observed signal. This decrease in signal is most notable for piracetam wherein at 70 °C or greater, the peak area goes to zero! This behavior can be explained using what has been termed as the "molar volatility factor" ($\Delta H_v/M$) [31]. It is hypothesized that piracetam has a smaller molar volatility factor leading to complete evaporation at temperatures higher than 70 °C. Similar observations have been documented by Charlesworth [31] and Trathnigg and Kollroser

[35]. The ratio of responses elicited by the analytes at lower temperatures (wherein no evaporation of the analyte is expected) cannot easily be explained since it depends on a variety of factors including size, shape, absorbance and surface properties of the particle and since equimolar concentrations of all analytes were not injected on to the column. However, constant *a* (as tabulated in Table 2) which is closely related to response factor f_i, suggests that equimolar quantities of piracetam, VPA-Na, PRM and CBZ will elicit similar responses.

3.2. Effect of carrier gas flow rate

The carrier gas flow rate was varied from 0.5 to 3.0 bar while maintaining all the other experimental parameters constant and data were collected pertaining to precision, response, separation and peak efficiency. As one would expect at lower carrier gas flow rates, the responses increased for all analytes. These results are plotted in Fig. 5. This can be qualitatively explained using Eq. (2). Slower gas flow rates (smaller Q_g) lead to an increase in the average droplet diameter, D_0, which in turn leads to a larger response. Coagulation of droplets, at slower gas flow rates, is another potential factor leading to an increase in D_0 [36]. The separation and peak efficiencies for all analytes decreased as the carrier gas flow rate was decreased. This is due to natural diffusion of one analyte into the other aided by the lack of purging by the carrier gas at low flow rates. One can correlate this observation to a similar situation in classic separation theory that causes a decrease in resolution at very low linear velocity due to back diffusion.

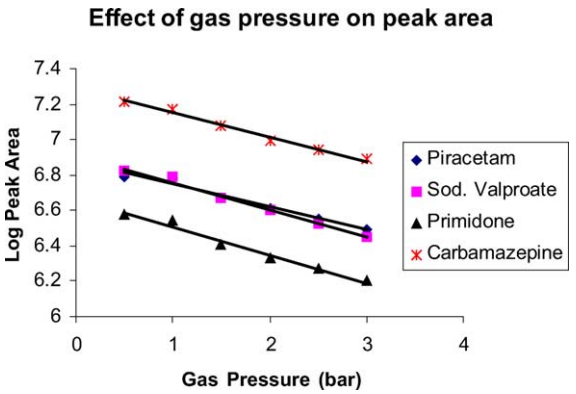

Fig. 5. Plot of log(peak area) of the four AEDs as a function of gas pressure.

3.3. Effect of buffer strength

It was observed that at buffer strengths equal to or greater than 0.03 M ammonium acetate, piracetam and VPA-Na co-eluted. It was also observed that at higher buffer strengths (>0.1 M), the reproducibility in peak areas of all four analytes were impacted. This is possibly due to incomplete and irreproducible evaporation of the buffer itself.

3.4. Effect of mobile phase flow rate

The mobile phase flow rate was varied in a narrow range of 0.3–0.7 ml/min. while maintaining all the other experimental parameters constant and data were collected pertaining to precision, response, separation and peak efficiency. A graph plotting responses (peak area) versus mobile phase flow rate is shown in Fig. 6. It is expected, based on Eq. (2), that at a constant gas flow rate (Q_g), the average droplet diameter (D_0) depends only on the mobile phase flow rate (Q_l) and increases to the power 1.5. This means that the intensity of scattered signal (as also the measured peak area) will increase with increasing mobile phase flow rate. However, from Fig. 6 it can be noted that the peak area responses are relatively same at all measured conditions. It is believed that this deviation from theory is due to the narrow range of mobile phase flow rates studied. There was no noticeable difference in separation or peak efficiency as a function of mobile phase flow rate.

3.5. Effect of photomultiplier gain

Photomultiplier gain was varied from 7 to 10 while maintaining all the other experimental parameters constant. The range between which the photomultiplier gain could be varied was primarily decided based on peak sensitivity (also reproducibility) and the maximum allowable input voltage (1024 mV) for the AD converter. A graph plotting log(peak area) and photomultiplier gain is shown in Fig. 7. It can be seen that at the gains studied all four analytes show a linear dependence. There was no noticeable difference in separation or peak efficiency as a function of photomultiplier gain.

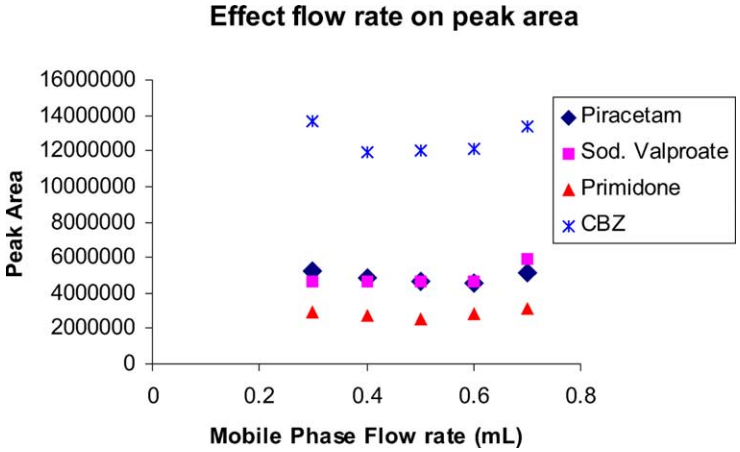

Fig. 6. Effect of mobile phase flow rate on peak area of the four AEDs.

3.6. Analyte linearity

A log(peak area) − log(mass) plot for all four analytes provided linear curves. Acceptable correlations have been obtained for all four analytes studied. The R^2 values, constants a and b for piracetam, VPA-Na, PRM and CBZ are given in Table 2. Satisfactory linearity of detector response has been simultaneously obtained for all four analytes.

3.7. Effect of column temperature

Column temperature was varied from 25 to 40 °C in 5 °C intervals while maintaining all the other experimental parameters constant. The effect of varying column temperature on the peak area response is plotted in Fig. 8. It can be seen that there is a slight increase in area for all analytes with increasing column temperature. This is most noticeable for CBZ. This increase in area is possibly due to better partitioning of the analyte in to the mobile phase at higher temperatures. This hypothesis is augmented by the noticeable increase in area for CBZ, the most hydrophobic drug of the four drugs under study. The increase in area as a result of better partitioning of the analyte in to the mobile phase is at least partially offset by the decrease in viscosity of the eluent as result of increased column temperature. There was no noticeable difference in

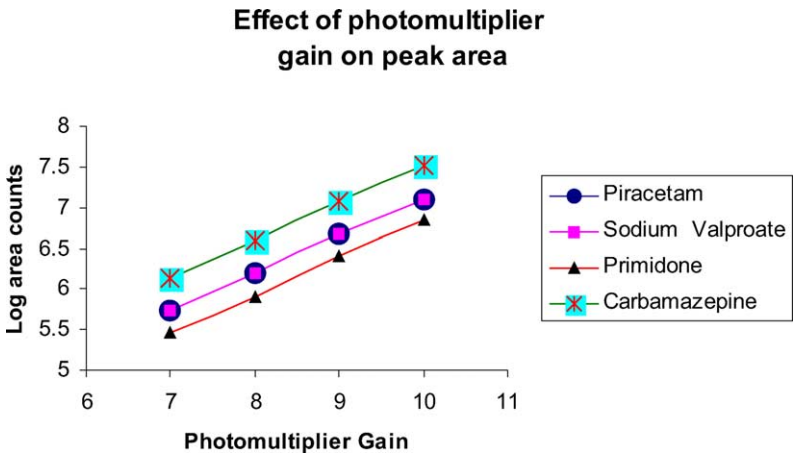

Fig. 7. A plot of log(area counts) of the four AEDs as a function on photomultiplier gain.

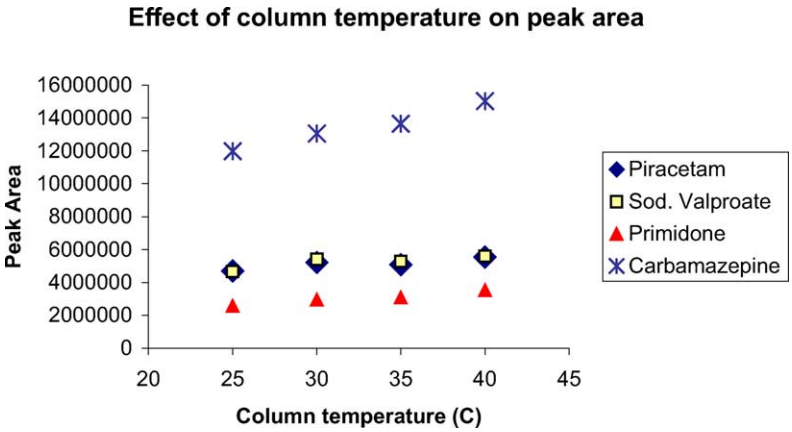

Fig. 8. A plot of the peak area of the four AEDs as a function of column temperature.

separation or peak efficiency as a function of column temperature.

The principles of separation, detection and quantitation of these four antiepileptic drugs, illustrated in non-bilological samples here, can be extended to analysis in biological specimens as well. Analysis of pharmaceutically relevant compounds in biological fluids using ELSD has been previously documented [22–25]. Sample preparation steps such as flow centrifugation, chemical deproteinization, liquid extraction, solid phase extraction and resin purification are performed on the biological sample to remove endogenous proteins prior to HPLC–ELSD. It is thus deemed that this method has potential application in drug level monitoring of patients undergoing mono- or polytherapy with any or all four of these drugs.

4. Conclusions

A novel, rapid, accurate, sensitive, reproducible and robust HPLC–ELSD method for the simultaneous separation and quantitation of four antiepileptic drugs has been developed. This method has been validated for precision, accuracy, linearity of detector response and robustness. In addition, the DL and QL for all four analytes have been determined.

In evaluating the effect of various critical experimental parameters on separation efficiency, accuracy, reproducibility and sensitivity of the method, it was observed that evaporator tube temperature was the single most important parameter directly related to peak parameters. It was noteworthy that at higher evaporator tube temperatures the peak area of piracetam decreased to zero due to lower molar volatility factor of piracetam. The physical basis of the observed results as a function of various critical experimental parameters has been explained using theories that have been previously developed.

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