Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, No. 5, pp. 431–436, 1990
Printed in Great Britain

0731–7085/90 $3.00 + 0.00
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Thin-layer chromatography and densitometry in drug assay: comparison of methods for monitoring valproic acid in plasma

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Abstract: A thin-layer chromatographic (TLC) method is described for the assay of the anti-epileptic drug, valproic acid, in plasma. The use of high-performance (HPTLC) plates gave sensitive detection limits (4.87 μg ml^{-1}) for derivatives of valproic acid and the reproducibility on the same or different plates was good. Comparison with high-performance liquid chromatography showed a similar performance of plate and column.

Keywords: *TLC densitometry; valproic acid; anti-epileptic agents.*

Introduction

Valproic acid (VPA), 2-propylvaleric acid, is one of the principal drugs used in the treatment of certain forms of epilepsy, especially the generalized primary forms (petit mal) and generalized tonic-clonic crises. It is usually given in combination with other drugs having analogous activity; effective plasma levels are 35–80 μg ml^{-1}.

Immunoenzymatic methods are most commonly used for the assay of VPA in biological fluids. Gas chromatography (GC) of the compound as such or in the form of its derivatives [1–8] is also used. A principal disadvantage of chromatographic methods is that the drug has to be isolated from the biological matrix and then detected. Immunoenzymatic and GC methods have been reported by Braun [8]. High-performance liquid chromatography (HPLC) has also been used in the assay of VPA [9, 10].

The aliphatic nature of the compound has permitted the development of methods of derivatization which enable the drug to be detected at suitable wavelengths. The most notable of these methods are based on the formation of phenacylic and naphthacylic esters.

No data have been published on the assay of VPA by thin-layer chromatography (TLC). In this paper a TLC method with densitometric detection is described for VPA in plasma. The comparative sensitivity and reproducibility of HPTLC and TLC and of HPLC and HPTLC together with the reproducibility of different HPTLC plates have been examined.

Experimental

Apparatus

For the densitometric measurements a CAMAG TLC scanning densitometer with a Hitachi Perkin–Elmer R100A recorder was used. For the HPLC investigations a Perkin–Elmer series 10 apparatus with an LC 85B spectrophotometric detector and a Perkin–Elmer R100A recorder were used.

A reversed-phase Perkin–Elmer HS C_{18} column was used.

Materials

HPTLC RP_8 254 S (10 × 20 cm), HPTLC Kiesel 60F254 (10 × 20 cm) and TLC C_8F octyl reversed-phase (20 × 20 cm) plates from Merck were used. The reagents, 2-naphthacyl bromide, 4-bromophenacyl bromide, dicyclohexane-18-crown-6-ether and VPA were from Aldrich Chemie; all other chemicals were analytical grade from Merck. Chromasolve solvents were from Riedel de Haen.

Quantitative application of solutions on the

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plates was performed with equilibrated Minicaps EM Hirschmann Labor Glass capillaries and a Hamilton 10-μl syringe was used for injection.

Derivatization procedure

The reagents used were 4-bromophenacylbromide and 2-naphthacyl bromide. Acetonitrile solutions of the former (20 mg ml^{-1}) and the latter (17 mg ml^{-1}) reagents contained 1 mg ml^{-1} of dicyclohexane-18-crown-6-ether as catalyst; the reagent–catalyst molar ratio was 25:1. Derivatization of VPA was performed with a buffer solution (0.35 M, pH 7.4) prepared by dissolving 3.8 g of potassium dihydrogen orthophosphate and 5.96 g of disodium hydrogen orthophosphate in 200 ml of water.

TLC conditions

Volume of solution applied 1 μl. Densitometric scanning: plate speed 0.5 mm s^{-1}; slit dimensions, width 0.3 mm and length 5.5 mm; detection wavelength 280 nm for the naphthacyl derivative and 254 nm for the phenacyl derivative; sensitivity 11. Recorder: range of potential 0.2 V; paper speed 20 mm min^{-1}. The silica 60 HPTLC plates were developed with chloroform–cyclohexane (2:1, v/v). For the HPTLC plates, ethanol–water (1:0.4, v/v) was used. For TLC, the best resolution was obtained with ethanol–water (1:0.6, v/v).

HPLC conditions

A Perkin–Elmer reversed-phase 3-μm C_{18} column (125 mm) was used with a 6-μl loop; the detection wavelength was 280 nm; isocratic elution was performed with methanol–water (1:0.33, v/v) at a flow-rate of 1 ml min^{-1}.

Extraction from water and plasma and derivatization

Fixed concentration solutions of VPA were prepared in 0.1 M potassium hydroxide; 0.2 ml of each solution was diluted with 0.5 ml of water, shaken and acidified with 0.3 ml of perchloric acid (25 m/m). The samples were then extracted with three 3-ml portions of cyclohexane, shaken in a Vortex mixer for 3 min and centrifuged for 5 min at 3500 rpm.

The pooled extracts were dried over sodium sulphate, treated with a methanolic solution of sodium methoxide (final content about 1 μmol of base), shaken for 1 min and dried under a stream of nitrogen at ambient temperature. The residue was redissolved in 0.2 ml of acetonitrile and treated with 0.1 ml of the buffer solution and 0.2 ml of the reagent solution. The mixture was incubated at 70°C for 40 min.

The same steps were followed for the extraction of plasma.

VPA regression curves

0.2 Millilitres of each of a set of standard solutions of VPA were added to 0.5 ml of water or plasma to give solutions of concentration 1–150 μg ml^{-1}. The solutions were extracted, dried and treated with the derivatizing reagent as described above. In parallel experiments, solutions of VPA with concentrations of 1–150 μg ml^{-1} were directly treated.

Recovery

The recovery was evaluated in six pools (three aqueous and three in plasma) of 10 samples each at VPA concentrations of 30, 70 and 100 μg ml^{-1}. Each sample was treated as described above. In parallel experiments, solutions of fixed VPA concentration which had not undergone extraction were treated.

Results and Discussion

The extraction conditions for VPA constitute one of the critical aspects of the assay of this compound in biological fluids. VPA can be satisfactorily extracted from acidic aqueous solution by apolar organic solvents (e.g. cyclohexane and pentane). This ensures the high selectivity of the process and drastically reduces the possible interference of endogenous compounds and of drugs such as those associated with the use of VPA in the treatment of epilepsy. There are certain problems in the concentration of the extract; part of the VPA may evaporate so that the recovery and reproducibility of the process are reduced. Microextraction and direct assay of the solution or derivatization and subsequent evaporation of the solvent have been proposed to obviate these difficulties. In the present study it was also observed that extraction with cyclohexane, evaporation of the solvent and then derivatization of the residue guaranteed high selectivity but compromised not so much the recovery as the reproducibility of the method. Using a pool of 10 extractions from water, the recovery was 80–95% but the relative standard

Table 1
Recovery of VPA from different matrices evaluated on HPTLC RP_8 plates

VPA added ($\mu g\ ml^{-1}$)	Water				Plasma			
	Mean recovery $\mu g\ ml^{-1}$ (mean %)	±SD	RSD (%)	R_M*	Mean recovery $\mu g\ ml^{-1}$ (mean %)	±SD	RSD (%)	R_M
30	26.50 (88.30)	1.443	4.81		25.20 (84.00)	1.041	3.47	
70	65.30 (93.31)	3.099	5.18	92.43	63.40 (90.70)	3.362	5.61	89.15
100	95.70 (95.70)	3.085	3.51		92.74 (92.74)	4.570	5.30	

*R_M = mean percentage recovery.

deviation (RSD) was high (10%). Extraction with cyclohexane was preferred; before evaporation of the organic solvent, a methanolic solution of sodium methoxide was added to prevent losses of VPA during evaporation by forming a salt. As shown in Table 1, in the range of concentrations usually found in the plasma of VPA-treated patients, the mean recovery was near 90% and the values for RSD were acceptable.

Of the reagents used for the spectrophotometry of aliphatic acids, 4-bromo-phenacyl bromide is the most common for HPLC of VPA. Only Alric *et al.* [11] used 2-naphthacyl bromide. Both reagents were investigated in the present work; better results were obtained with the latter reagent. 2-Naphthacyl bromide, unlike the phenacyl derivative, gave a bland blue fluorescence with Wood's lamp and this enabled as little as 10 ng of VPA to be detected visually. The derivatization conditions of Alric *et al.* [11] were followed but phosphate buffer (pH 7.4, 0.35 M; as used by other authors for the phenacyl derivative) was employed instead of the bicarbonate buffer which resulted in the formation of other spots with the reagent also complicated resolution. It was found that aliphatic acids such as lactic, pyruvic, methylmalonic, aminobutyric and hydroxybutyric did not affect the VPA spot.

Different types of stationary phase were tried with HPTLC and TLC plates. C_{18} columns in HPLC were used to compare the performance of the two types of chromatographic system. Silica 60 plates were found to be unsatisfactory for matrices of plasma as shown in Fig. 1. In fact, an unidentified plasma component had an R_f value which exactly coincided with that of VPA. By extracting VPA from aqueous solution, satisfactory results in terms of the statistical parameters of

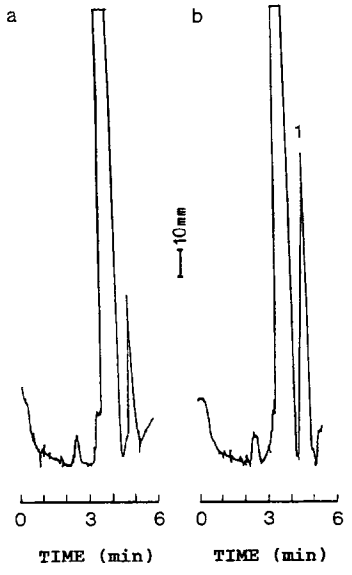

Figure 1
Chromatographic resolution on silica 60 HPTLC plates. (a) Plasma extract before addition of VPA (1 µl applied). (b) Plasma extract after addition of VPA (1 µl containing 80 ng VPA applied). VPA = 1. Mobile phase:chloroform–cyclohexane (2:1, v/v).

the regression curve and sensitivity of response were obtained (Table 2). Detection limits in plasma of 9.70, 4.87 and 3.47 $\mu g\ ml^{-1}$ were found, respectively, for the TLC RP_8 plates, HPTLC RP_8 plates and HPLC.

The best chromatographic resolution was obtained with C_8 reversed-phase plates as shown in Figs 2 and 3.

The regression lines, calculated from the ratio of peak height to drug content, had a greater slope for the HPTLC RP_8 plates than for the TLC RP_8 plates; the lower detection limits for HPTLC RP_8 plates were about one-half those for TLC RP_8 plates. This may be seen in Table 2. Differences between the slopes were much less when regression line

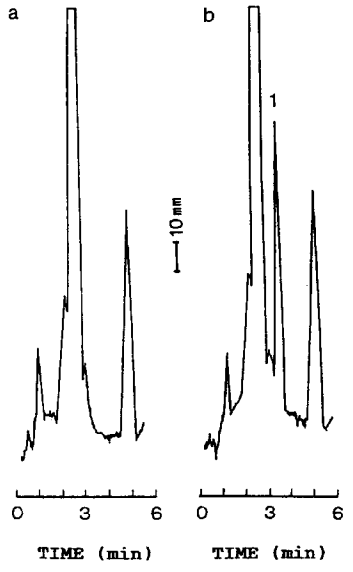

Figure 2
Chromatographic resolution on HPTLC RP_8 plates. (a) Plasma extract before addition of VPA (1 μl applied). (b) Plasma extract after addition of VPA (1 μl containing 80 ng VPA applied). VPA = 1. Mobile phase: ethanol–water (1:0.4, v/v).

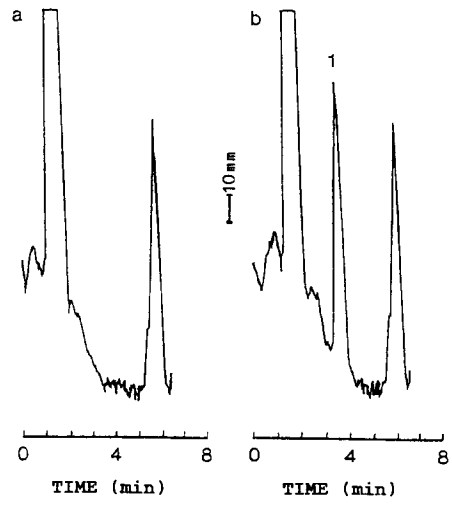

Figure 3
Chromatographic resolution on TLC RP_8 plates. (a) Plasma extract before addition of VPA (1 μl applied). (b) Plasma extract after addition of VPA (1 μl containing 80 ng VPA applied). VPA = 1. Mobile phase:ethanol–water (1:0.6, v/v).

Table 2
Regression line parameters of VPA* for different chromatographic conditions

Type of analysis	System analysed	Intercept (mm)	Slope (mm ml μg^{-1})	Correlation coefficient	Lower detection limit ($\mu g\ ml^{-1}$)†
HPTLC RP_8	Direct	0.651	2.124	0.996	3.97
	Water	−0.012	1.815	0.998	4.56
	Plasma	−0.276	1.783	0.944	4.87
TLC RP_8	Direct	0.020	1.970	0.999	6.20
	Water	0.960	1.556	0.998	8.65
	Plasma	0.477	1.500	0.996	9.70
HPTLC Si 60	Direct	−0.320	2.350	0.997	2.30
	Water	−0.152	1.968	0.991	3.17
HPLC C_{18}	Direct	0.861	1.941	0.996	1.58
	Water	0.242	1.686	0.998	2.64
	Plasma	−0.234	1.696	0.998	3.47

*Range of calibration 1–150 $\mu g\ ml^{-1}$.
†Lower detection limit = $3N/m$, where m = slope of regression line and N = height (mm) of background noise.

Table 3
Regression lines parameters of VPA* based upon peak area measurements on different RP_8 plates

Type of analysis	System analysed	Intercept (mm)	Slope (mm ml μg^{-1})	Correlation coefficient
HPTLC RP_8	Direct	−0.319	4.719	0.994
	Water	−0.734	3.965	0.997
	Plasma	0.096	3.815	0.997
TLC RP_8	Direct	0.875	4.918	0.991
	Water	0.339	4.112	0.994
	Plasma	−0.367	3.999	0.998

*Range of calibration 1–150 $\mu g\ ml^{-1}$.

values based on peak area were compared (Table 3).

The reproducibility of HPTLC RP_8 and TLC RP_8 plates was compared by applying 10 spots of a plasma extract on four plates of each type. The RSD was calculated for each of the eight plates and this parameter was used for Student-*t* analysis of the results for the two types of layers. No statistically significant difference was found ($P < 0.1$).

It seems logical to conclude that on HPTLC plates there is more uniform stratification which guarantees less background noise, and that the spots are more homogeneously distributed in a smaller area. The densitometric findings are thus characterized by narrower and higher peaks on high-performance plates. It was even possible to appreciate visually the different dimensions and intensity of the spots on TLC and HPTLC plates.

For HPLC, the optimal mobile phase was methanol–water (77:23, v/v). The regression line parameters are shown in Table 2 and the resolution is shown in Fig. 4.

The results obtained by TLC and column chromatography were then compared. As the values obtained in the HPLC study agree with those of other workers, the results of the comparison may be extended to the general case.

The lower detection limit for plasma samples for the HPLC assay was 3.47 μg ml^{-1} and for the HPTLC assay was 4.87 μg ml^{-1}.

The reproducibility of HPLC determined by injecting the same solution 10 times was good with a RSD of 3.57% at a concentration of 60 μg ml^{-1}.

As the performance of HPTLC plates is better than that of TLC plates in terms of time (elution and densitometric scanning times) and sensitivity based on peak height, the reproducibility between plates of the same and different lots were determined. With three different lots a derivatized solution, extracted from plasma, was applied on six plates (two from each lot).

The findings for each pair were analysed by the Student-*t* test. Variance analysis was applied to results from the six plates. Both evaluations showed that the differences were not statistically significant for $P < 0.01$.

Finally, four tests on plasma from patients undergoing combined antiepileptic therapy with VPA, carbamazepine and phenobarbitone were performed. The results were comparable with those determined by immunoenzymatic methods; the maximum percentage difference between the results of the two methods was 5.85%.

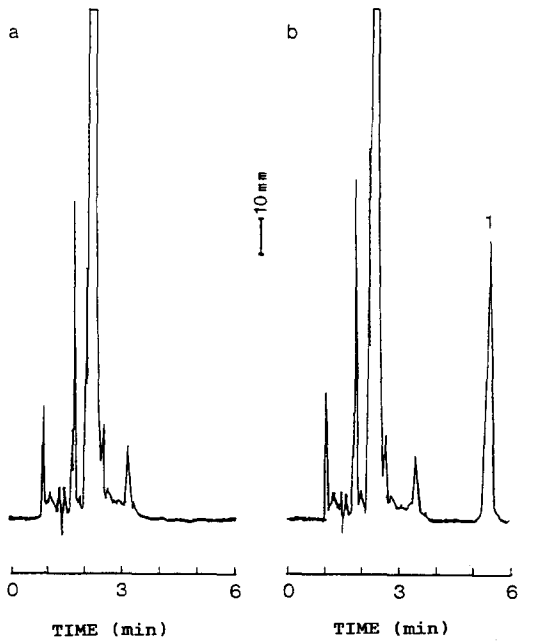

Figure 4
High-performance liquid chromatographic resolution. (a) Plasma extract before addition of VPA. (b) Plasma extract after addition of VPA. VPA = 1. Chromatographic conditions: fixed phase = HS C_{18} Perkin Elmer; mobile phase = methanol–water (77:23, v/v); flow-rate = 1 ml min^{-1}.

Conclusion

From the point of view of performance, economy, simplicity and reproducibility, TLC with high-performance reversed-phase plates is a valid alternative method of assaying VPA. There were no significant differences in sensitivity and reproducibility from results obtained by column chromatography. TLC plates also gave good results but were slower than HPTLC plates.

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[Received for review 19 February 1988; revised manuscript received 16 April 1990]