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ANALYTICAL ASPECTS OF BARBITURATE ABUSE

IDENTIFICATION OF DRUGS BY THE EFFECTIVE COMBINATION OF GAS–LIQUID, HIGH-PERFORMANCE LIQUID AND THIN-LAYER CHROMATOGRAPHIC TECHNIQUES

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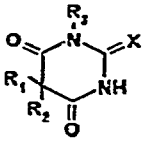
SUMMARY

Chromatographic retention data for a group of barbiturates have been measured on seven chromatographic systems (two gas–liquid chromatographic (GLC), three high-performance liquid chromatographic (HPLC) and two thin-layer chromatographic systems) and the value of these for barbiturate identification has been discussed. The overall correlations observed between pairs of systems are generally low; however, specific groups of barbiturates show very high correlations and this determines the approach to the selection of two or more systems to increase chromatographic discrimination of the barbiturate group. Column chromatographic techniques with lipophilic phases (GLC using SE-30, HPLC using ODS-silica) are most suitable for barbiturate identification. Changes of eluent pH in reversed-phase HPLC proved very effective for the separation of barbiturates with closely related structures.

INTRODUCTION

It has been estimated that of over 2500 barbiturates which have been synthesised more than 50 of these are presently marketed for clinical use throughout the world. Drug abuse involving barbiturates is widespread and the international nature of the illegal markets means that any forensic laboratory may encounter a vast range of these compounds. Furthermore, the abused barbiturates often occur in mixtures with other barbiturates, other drugs and/or excipients. The isolation and identification of a specific barbiturate thus poses a considerable analytical problem.

The barbiturates are all derivatives of barbituric acid ($R_1 = R_2 = R_3 = H$; $X = O$) and those with clinical use fall into three groups: 5,5-disubstituted oxybarbi-



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turic acids (R_1, R_2 = alkyl or aryl; R_3 = H; X = O); 1,5,5-trisubstituted oxybarbituric acids (R_1, R_2 = alkyl or aryl; R_3 = alkyl; X = O); 5,5-disubstituted thiobarbituric acids (R_1, R_2 = alkyl or aryl; R_3 = H; X = S).

All three groups of barbiturates can be distinguished by the nature of their ultraviolet (UV) spectra with changes in pH (ref. 1); however, the identification of individual compounds within any group is not possible using this method. Furthermore, UV spectroscopy may not be possible if the barbiturates are present in a mixture. The use of chromatographic techniques for the isolation and identification of barbiturates is clearly desirable. Although many procedures for the chromatographic separation of barbiturates have been published, the principles of selecting the most efficient systems (or combinations of systems) have not been discussed.

Of the clinically useful barbiturates the thiobarbituric acids have a rapid onset of action and are used almost exclusively as anaesthetics. The oxybarbituric acids are generally slower acting drugs and are used as sedative-hypnotics or anticonvulsants; these compounds are more likely to be abused. In the following study chromatographic data are presented for 28 oxybarbiturates which are likely to be encountered in forensic and clinical laboratories. A comparison of the separation of these barbiturates by gas–liquid chromatography (GLC), high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) systems has been made and the combination of these systems to give maximum discrimination has been investigated.

EXPERIMENTAL

GLC was performed on a Pye 104 gas chromatograph fitted with a flame ionization detector and a glass column (2 m × 4 mm I.D.) packed with 3% SE-30 on Chromosorb G HP (80–100 mesh). Nitrogen flow-rates of 45–50 ml/min with oven temperatures of 190–200°C were used. Barbiturates were injected as solutions in ethanol. On-column methylation was carried out with Methelute (Pierce and Warriner, Chester, Great Britain).

HPLC was performed with a constant-flow pump (Waters M6000), a variable-wavelength UV detector (Pye-Unicam LC-UV) and a valve injector (Rheodyne 7120) fitted with a 20-μl loop. The normal-phase column (250 × 4.6 mm I.D.) and the reversed-phase column (150 × 4.6 mm I.D.) were packed with Hypersil and ODS-Hypersil (Shandon Southern Products, Runcorn, Great Britain) respectively. The reversed-phase eluents were prepared by mixing aqueous sodium dihydrogen phosphate (0.1 *M*) and methanol then adjusting the final pH with sodium hydroxide or phosphoric acid. Flow-rates of 2 ml/min were used throughout. The detector was operated at 216 nm for reversed-phase and 250 nm for normal-phase chromatography. Barbiturates were injected in ethanolic solution.

TLC was conducted using glass-coated silica gel 60 F254 plates, 20 × 20 cm, 0.25 mm thickness from E. Merck (Darmstadt, G.F.R.). The spots were detected by spraying with ethanolic diphenylcarbazone and mercuric chloride[2].

The seven chromatographic systems examined in this study were as follows:

(1) GLC using SE-30 stationary phase;

(2) GLC using SE-30 stationary phase with co-injection of Methelute;

(3) HPLC using ODS-silica with an eluent of 40% methanol at pH 3.5;

(4) HPLC using ODS-silica with an eluent of 40% methanol at pH 8.5;

(5) HPLC using silica with an eluent of isooctane–acetic acid–isopropanol (200:3:2, v/v/v);

(6) TLC using silica plates with a developing solvent of chloroform–acetone (4:1, v/v);

(7) TLC using silica plates with a developing solvent of isopropanol–chloroform–ammonia (9:9:2, v/v/v).

RESULTS AND DISCUSSION

Choice of chromatographic systems

Barbiturates have been chromatographed using a wide range of GLC stationary phases and Berry[3], considering the needs of the clinical toxicologist, has recommended the CDMS phase. The choice of SE-30 in the present work reflects the wide application of this phase to forensic problems and the vast amount of data on drugs which is already available[4,5]. Other GLC stationary phases are certainly capable of providing satisfactory separations of the barbiturates.

Hydrocarbonaceous bonded phases have been widely used in HPLC for the separation of pharmaceutical compounds including small groups of barbiturates. Separations using ODS-silica[6–12], SAS-silica[13] and methyl-silica[14] have been reported and the limited data available for aqueous methanolic eluents show a common order of elution and suggest that similar mechanisms are operating on these phases. Initial experiments showed that differences in selectivity could be achieved by changing the pH of the eluent. Two of the systems examined involve the chromatography of barbiturates on ODS-silica. Both eluents contain 40% methanol and the two differ only in the pH. Only a few publications give data concerning the separation of barbiturates by HPLC using microparticulate silica[15–19]. Our initial experiments showed that the barbiturates give poor peak shapes with silica when neutral or basic eluents were used. A system was subsequently developed using an acidic eluent which gave good peak shapes and efficiencies.

Many TLC systems for the separation of barbiturates have been recorded in the literature[20,21]. The two systems included in this study were chosen as examples of systems frequently used in forensic casework[2]. One involves a neutral developing solvent (chloroform–acetone) and the other, a basic solvent (isopropanol–chloroform–ammonia).

The chromatographic retention data for the 28 barbiturates on the seven system are given in Table I.

Comparison of the chromatographic systems

Any chromatographic system which is proposed as a routine analytical procedure must fulfill several requirements: the analysis time must be as short as possible; the system should be simple to set up (*e.g.*, isothermal GLC, isocratic HPLC); the system must achieve the necessary level of reproducibility both within-laboratory and between-laboratory; the detection method for the system must be sufficiently sensitive for the application. Any chromatographic system which meets these general criteria must then be examined with reference to the separation of the group of compounds of interest.

TABLE I

CHROMATOGRAPHIC RETENTION DATA FOR 28 BARBITURATES

System 1, GLC using SE-30; system 2, GLC using SE-30 with methylation; system 3, HPLC using ODS-silica (pH 3.5); system 4, HPLC using ODS-silica (pH 8.5); system 5, HPLC using silica; system 6, TLC using silica (chloroform–acetone); system 7, TLC using silica (isopropanol–chloroform–ammonia).

Barbiturate	*Structure*			*GLC (RI)*		*HPLC (k')*			*TLC ($R_F \times 100$)*	
	R_1	R_2	R_3	*System 1*	*System 2*	*System 3*	*System 4*	*System 5*	*System 6*	*System 7*
Allobarbitone	Allyl	Ally	H	1586	1491	2.46	1.33	9.03	59	53
Amylobarbitone	Ethyl	Isopentyl	H	1700	1600	10.91	7.05	7.17	66	74
Aprobarbitone	Isopropyl	Allyl	H	1600	1513	3.42	2.22	8.10	59	66
Barbitone	Ethyl	Ethyl	H	1482	1415	1.11	0.63	11.95	51	51
Brallobarbitone	Allyl	2-Bromoallyl	H	1842	1741	3.09	1.72	10.88	60	47
Butalbital	Isobutyl	Allyl	H	1658	1553	6.17	3.48	6.68	63	67
Butobarbitone	Ethyl	*n*-Butyl	H	1645	1557	5.43	3.42	7.60	64	68
Cyclobarbitone	Ethyl	Cyclohex-1-enyl	H	1945	1850	5.25	2.61	10.04	59	59
Cyclopentobarbitone	Allyl	Cyclopent-2-enyl	H	1858	1751	6.00	3.84	8.13	64	62
Enallylpropymal	Isopropyl	Allyl	Methyl	1559	1522	8.65	6.96	1.90	88	87
Heptabarbitone	Ethyl	Cyclohept-1-enyl	H	2035	1932	9.90	4.93	9.42	61	62
Hexethal	Ethyl	*n*-Hexyl	H	1835	1748	34.28	20.39	6.31	71	74
Hexobarbitone	Methyl	Cyclohex-1-enyl	Methyl	1850	1800	7.37	5.67	4.87	77	85
Ibomal	Isopropyl	2-Bromoallyl	H	1866	1759	4.01	2.58	10.27	63	61
Idobutal	*n*-Butyl	Allyl	H	1698	1608	8.12	4.77	6.33	68	71
Metharbitone	Ethyl	Ethyl	Methyl	1450	1410	2.69	1.99	2.75	79	86
Methohexitone	Allyl	1-Methylpent-2-ynyl	Methyl	1768	1721	27.61	20.48	1.48	88	93
Methylphenobarbitone	Ethyl	Phenyl	Methyl	1882	1832	7.27	3.84	2.91	80	72
Nealbarbitone	2,2-Dimethylpropyl	Allyl	H	1720	1609	10.22	6.19	5.79	69	78
Pentobarbitone	Ethyl	1-Methylbutyl	H	1733	1632	10.96	8.07	6.78	65	76
Phenobarbitone	Ethyl	Phenyl	H	1934	1831	3.09	1.23	12.57	56	38
Phenylmethylbarbituric acid	Methyl	Phenyl	H	1875	1789	1.48	0.94	24.20	41	27
Probarbitone	Ethyl	Isopropyl	H	1550	1480	2.15	1.57	8.96	57	65
Quinalbarbitone	1-Methylbutyl	Allyl	H	1770	1670	16.28	11.47	5.63	72	78
Secbutobarbitone	Ethyl	1-Methylpropyl	H	1650	1564	4.89	3.32	7.59	60	69
Sigmodal	1-Methylbutyl	2-Bromoallyl	H	2031	1901	19.58	12.37	5.87	73	73
Talbutal	1-Methylpropyl	Allyl	H	1704	1592	7.25	4.67	6.50	65	71
Vinbarbitone	Ethyl	1-Methylbut-1-enyl	H	1755	1650	4.83	2.32	9.88	62	56

The effective separation of a given group of compounds with a chromatographic system is controlled by two factors. Firstly, the band spreading which occurs as the compounds migrate through the stationary phase determines the number of compounds which can be resolved across the chromatographic range (*e.g.*, the number of spots which can be separated across a TLC plate). Minimum band spreading is clearly desirable and for column chromatography (GLC, HPLC) this is expressed as a high plate count. No attempt has been made in this study to optimise this factor for the seven chromatographic systems. In general, the GLC and HPLC systems are capable of resolving more compounds than the TLC systems. Longer GLC or HPLC columns would give greater plate counts but only at the cost of longer analysis times. The second factor which determines the effectiveness of a system concerns the frequency distribution of the compounds across the chromatographic range. Maximum discrimination occurs when the retention parameters for the group show an even distribution over the entire range.

The frequency distributions for the retention parameters of the 28 barbiturates on the GLC, HPLC and TLC systems are shown in Fig. 1. The retention index (RI) values for the barbiturates with SE-30 stationary phase show a good distribution over the chromatographic range (Fig. 1a); no more than four compounds lie within any range of 50 RI units. Comparison of these results with those for GLC with methylation (Fig. 1b) shows an overall move towards smaller RI values with a slight contraction of the distribution. This is expected as the addition of methyl groups increases the volatility of the barbiturates. Opposed to this, methylation gave an improvement in peak symmetry for most compounds. Two peaks were observed when one of the barbiturates containing a 2-bromoallyl substitutent (brallobarbitone, ibomal and sigmodal) was co-injected with Methelute. The peak with the longer retention time represented the dimethyl derivative while the other peak resulted from the elimination of hydrogen bromide from this derivative. Peaks were identified by mass spectrometry.

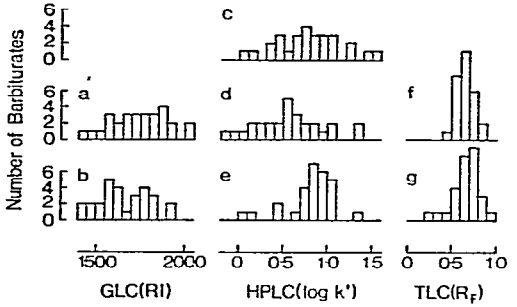

Fig. 1. Frequency distributions of retention parameters for 28 barbiturates on 7 chromatographic systems: (a) GLC using SE-30; (b) GLC using SE-30 with methylation; (c) HPLC using ODS-silica at pH 3.5; (d) HPLC using ODS-silica at pH 8.5; (e) HPLC using silica; (f) TLC using silica plates with chloroform–acetone; (g) TLC using silica plates with isopropanol–chloroform–ammonia.

Figs. 1c and 1d show the frequency distributions for the reversed-phase HPLC systems which differ only by the pH of the eluent. The data in Fig. 1c (pH 3.5) show a shift towards longer retention times relative to those in Fig. 1d (pH 8.5) and reflect the increase in lipophilicity of the barbiturates as they change from the ionised to the

TABLE II

LINEAR CORRELATION COEFFICIENTS FOR THE COMBINATION OF RETENTION DATA FROM PAIRS OF CHROMATOGRAPHIC SYSTEMS

Calculations involving GLC and TLC data use RI and R_f values respectively. Combinations of HPLC data with other HPLC data use k', combinations with GLC data use log k' and combinations with TLC data use R_f values; $R_F = 1/(1 + k')$ (ref. 24).

Chromatographic system combinations	*Linear correlation coefficients (r)*		
	All barbiturates (28)	*5,5-Dialkyl barbituric acids (7)*	*5-Alkyl-5-allyl barbituric acids (6)*
a GLC (SE-30) *vs.* HPLC (ODS-silica; pH 3.5)	0.379	0.995	0.986
b GLC (SE-30) *vs.* HPLC (silica)	0.216	0.944	0.959
c GLC (SE-30) *vs.* TLC (chloroform–acetone)	0.050	0.967	0.919
d HPLC (ODS-silica; pH 3.5) *vs.* HPLC (silica)	0.557	0.935	0.969
e HPLC (ODS-silica; pH 3.5) *vs.* TLC (chloroform–acetone)	0.673	0.968	0.965
f HPLC (silica) *vs.* TLC (chloroform–acetone)	0.901	0.965	0.967
g GLC (SE-30) *vs.* GLC (SE-30 with methylation)	0.989	0.997	0.989
h HPLC (ODS-silica; pH 3.5) *vs.* HPLC (ODS-silica; pH 8.5)	0.986	0.997	0.992
i TLC (chloroform–acetone) *vs.* TLC (isopropanol–chloroform–ammonia)	0.876	0.898	0.912

neutral form. Nevertheless, both reversed-phase systems do show a wide distribution of data. Fig. 1e shows that the distribution across the normal-phase HPLC system is not so even with a concentration of data around $k' = 7$.

The frequency distributions of the R_F values for the two TLC systems are shown in Figs. 1f and 1g; considerable clustering can be seen in both systems. The chloroform–acetone system (Fig. 1f) has 25 barbiturates with R_F values between 0.5 and 0.8 while the isopropanol–chloroform–ammonia system has only a slightly better distribution with 21 drugs within this range.

It is clear that the two GLC systems and the two reversed-phase HPLC systems have better distributions of chromatographic retention parameters than any of the systems based on silica as the stationary phase (*i.e.*, the normal-phase HPLC system and the two TLC systems). The lipophilic nature of the SE-30 and ODS-silica stationary phases thus provides a better separation of the group of barbiturates. This reflects the fact that the barbiturates differ from each other by the nature of their lipophilic substituents. Of the three chromatographic systems based on silica (two TLC systems and one HPLC system) the normal-phase HPLC system shows the best frequency distribution; furthermore it is capable of resolving a greater number of compounds across its chromatographic range.

Combination of chromatographic systems

The number of compounds which can be resolved on a single chromatographic system is limited and hence the combination of data from different systems to give extra discrimination is desirable. It is generally regarded that the combination of chromatographic systems to give maximum discrimination requires a low correlation between the systems[22]. An examination of the relationships between the present experimental systems has therefore been performed (see Table II). Although the overall correlations between pairs of systems for the 28 barbiturates may be low, specific groups of barbiturates show very high correlations.

This is well illustrated by the combination of the GLC data obtained for the barbiturates using SE-30 and the reversed-phase HPLC data at pH 3.5. The 28 barbiturates show a low overall correlation between these two systems ($r = 0.379$); however, the dialkylbarbituric acids and alkyl, allylbarbituric acids show high linear correlations ($r = 0.995$ and $r = 0.986$ respectively). The regression lines for these groups are parallel and very close together (Fig. 2; lines C and D). The dialkylbarbituric acids and the alkyl, allylbarbituric acids can be combined with allobarbitone (5,5-diallylbarbituric acid) with no significant change in the overall linear correlation coefficient ($r = 0.992$). It thus appears that an allyl group contributes to the chromatographic retention properties of the barbituric acids on these two systems in a similar way to an alkyl group.

Fig. 2 shows four other lines drawn parallel to lines C and D; these represent further specific groups of barbiturates. Line A passes through enallylpropymal (1-methyl-5-isopropyl-5-allylbarbituric acid); line B passes through metharbitone (1-methyl-5,5-diethylbarbituric acid); line E passes through the two alkyl, bromoallylbarbituric acids (ibomal and sigmodal); line F passes through the two alkyl, phenylbarbituric acids (methylphenobarbitone and phenobarbitone). The proximity of line A (1-alkyl-5-alkyl-5-allyl barbiturates) to line B (1-alkyl-5,5-dialkyl barbiturates) and the observation that brallobarbitone (an allyl, bromoallylbarbituric

acid) falls close to line E (the alkyl, bromoallylbarbituric acids) give further evidence that an allyl group contributes to the GLC and HPLC retention properties in a very similar way to an alkyl group.

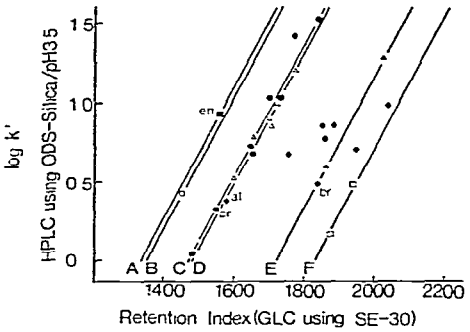

Fig. 2. Correlation of GLC retention data (SE-30 stationary phase) for 28 barbiturates with HPLC retention data (ODS-silica; eluent pH 3.5). ■ = 1-methyl-5-alkyl-5-allylbarbituric acids (line A); ○ = 1-methyl-5,5-dialkylbarbituric acids (line B); ● = 5,5-dialkylbarbituric acids (line C); △ = 5-alkyl-5-allyl-barbituric acids (line D); ▲ = 5-alkyl-5-bromoallylbarbituric acids (line E); □ = 5-alkyl-5-phenylbarbituric acids (line F); ◈ = all other barbiturates. al = allobarbitone, br = brallobarbitone, en = enallylpropymal and pr = probarbitone.

Table II gives correlation data for combinations of the experimental chromatographic systems. Table II (a–f) represents the possible combinations of the GLC system without methylation, the reversed-phase HPLC system at pH 3.5, the normal-phase HPLC system and the TLC system with chloroform–acetone developing solvent. Linear correlation coefficients have been calculated for the total group of 28 compounds as well as the dialkylbarbituric acids and the alkyl, allylbarbituric acids for each pair of systems. The linear correlation coefficients for the total group of barbiturates are generally low, the highest value being observed for the combination of the normal-phase HPLC and TLC systems ($r = 0.901$). The sub-division of the 28 barbiturates into structurally related groups leads to a large increase in correlation coefficient in all cases. The lowest linear correlation for the group of dialkylbarbituric acids is observed for the combination of the normal-phase and reversed-phase HPLC systems ($r = 0.935$); however, these data points lie on a smooth curve and hence calculation of a linear correlation coefficient cannot totally reflect the inter-relationship between the two sets of data. Similar non-linear correlations are observed with other combinations of systems.

The combinations of systems already considered represent different modes of chromatography (*e.g.*, HPLC using silica *vs.* TLC using silica) or different stationary phases (*e.g.*, HPLC using silica *vs.* HPLC using ODS-silica). Table II (g–i) gives combinations of systems involving the same mode of chromatography with identical stationary phases. The data show that the overall correlations for these pairs are high, however sub-division of the barbiturates into structurally related groups still leads to an increase in correlation in most cases. A plot showing the combination of the two GLC systems shows that the data points for the group of disubstituted barbiturates fall around one line ($r = 0.997$) while the trisubstituted barbiturates fall on a separate but parallel line ($r = 1.000$).

The identification of an unknown compound using chromatographic techniques involves the unique matching of the chromatographic properties of the compound with a single member of a defined group of compounds. Such a unique match differentiates the unknown from other members of the defined group but does not exclude compounds from outside the group. If an unknown compound does not show a unique match with the first chromatographic system (*i.e.*, the unknown compound shows identical chromatographic properties with a group of unresolved compounds within the group) a second system must be chosen which can separate these unresolved compounds. The high correlations for structurally related groups of barbiturates between different chromatographic systems which have been observed in this study must influence the choice of the second chromatographic system. It can be concluded that the second system must be chosen with reference to the nature of the compounds which need to be separated and not on the overall correlation of the two systems. If the unresolved barbiturates belong to different structural groups the second system should be selected such that the combination gives maximum separation of the regression lines for the structurally related groups. Enallylpropymal (a trisubstituted barbituric acid) and probarbitone (a disubstituted barbituric acid) provide an example of this type. The free barbiturates are not resolved by GLC (SE-30) but are separated from the remaining 26 barbiturates in the group. The correlation of the GLC data with the data from reversed-phase HPLC at pH 3.5 shows that the regression lines on which the data points for the two compounds fall are well separated (Fig. 2). The reversed-phase HPLC system is therefore a good choice for the second chromatographic system and enables these compounds to be separated. Similarly, the two compounds lie on well separated regression lines when the two GLC systems are correlated and hence GLC using SE-30 with methylation would also be a satisfactory second system.

Alternatively, when the barbiturates not resolved on the first chromatographic system are of the same structural type (*e.g.*, both dialkylbarbituric acids) the second system must be chosen such that the correlation of this structural group between the two systems is as low as possible. The separation of such compounds is often difficult, *e.g.*, butobarbitone and secbutobarbitone can only be separated by reversed-phase HPLC at pH 3.5. The use of the two reversed-phase HPLC systems (one at pH 3.5, the other at pH 8.5) in combination proved very useful for the separation of such difficult pairs. The pK_a values of the barbiturates fall within the range 7–8.5 (ref. 23) and therefore the compounds are fully protonated at pH 3.5 but only partially so at pH 8.5. Although the overall correlation between these two systems is high (Table II, h) many changes in selectivity occur between them, *e.g.* amylobarbitone–pentobarbitone and cyclobarbitone–butobarbitone pairs can be resolved at pH 8.5 but not at pH 3.5 while amylobarbitone–enallylpropymal, butobarbitone–secbutobarbitone and cyclobarbitone–vinbarbitone pairs can be resolved at pH 3.5 but not at pH 8.5.

In conclusion, the present study has shown that TLC retention data have limited value for the identification of barbiturates although it should be remembered that the application of specific visualisation reagents (*e.g.* potassium permanganate for barbiturates with unsaturated substituents) can give some increase in discrimination. The column chromatographic systems with lipophilic stationary phases (GLC using SE-30, HPLC using ODS-silica) are to be preferred as they show a wide distribution of retention parameters. The effective combination of different chromato-

graphic systems to increase discrimination must consider the high correlations observed for groups of structurally related barbiturates and should not be based on the overall correlation of the retention data between the systems. The separation of barbiturates with very similar structures is best approached by using the effect of eluent pH on reversed-phase HPLC.

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