JOURNAL OF
CHROMATOGRAPHY B

ELSEVIER Journal of Chromatography B, 694 (1997) 99–114

Development of a high-performance liquid chromatography retention index scale for toxicological drug screening

Simon P. Elliott*, Keith A. Hale

Regional Laboratory for Toxicology City Hospital N.H.S. Teaching Trust, Dudley Road, Birmingham B18 7QH, UK

Received 17 September 1996; revised 14 January 1997; accepted 27 January 1997

Abstract

An efficient and practical analytical method for correcting HPLC retention data has been produced using an HPLC diode-array UV detector system. The system is based on retention indices (RI) and is to be used primarily for the identification of toxicologically relevant drugs involved in clinical and forensic toxicology. The RI correction method was chosen as it provided a slightly greater degree of reproducibility than using relative retention time (RRT), particularly for acidic and neutral drugs. Development of the system involved the establishment of the optimal chromatographic conditions and extensive studies of the stability of the system. An acetonitrile gradient elution was used with RI values determined by interpolation from a series of specifically chosen basic and acidic/neutral marker drugs which eluted at regular intervals to produce a linear RI scale. It was found that two separate RI scales were required for basic and acidic/neutral drugs. The use of multiple drug markers as primary retention index standards had not been previously applied to HPLC general drug screening and comparison with a recently published database suggests that the system may also provide improved selectivity.

Keywords: Toxicol drugs

1. Introduction

The analysis of biological fluids or tissues for an unknown drug or poison is one of the most challenging tasks presented to the clinical or forensic toxicologist. Chromatographic procedures, notably gas and thin-layer chromatography, usually comprise a major part of analytical schemes for such "unknown screens"; commonly, chromatographic retention parameters and detection signals are compared with databases generated from standard reference compounds. Such data-matching approaches can screen for and identify a large number of relevant substances, while providing results rapidly enough to be applicable in emergency clinical toxicology. Gas chromatography (GC) on modern fused silica bonded-phase capillary columns produces highly reproducible retention data which is suitable for compilation into large databases and possible exchange between laboratories [1–3]. Data from standardised thin-layer chromatography (TLC) has also been published [4–6], although the reproducibility of this technique is considerably poorer than capillary gas chromatography.

As high performance liquid chromatography (HPLC) equipment became widely available during the 1970s, many toxicology laboratories adopted it for (quantitative) analyses which were difficult or impossible by GC methods. Despite the advances made in GC instrumentation and columns during the

*Corresponding author.

PII S0378-4347(97)00095-9

last decade, many drugs, chemical poisons and their metabolites are either too polar, non-volatile (e.g., glucuronides, phenoxyacid herbicides), or thermally labile (e.g., carbamazepine, propoxyphene) to be analysed satisfactorily by GC without derivatisation.

Although HPLC generally provides much lower resolution than capillary GC, it has the potential to form the basis of screening systems which cover a wider range of toxicologically relevant compounds. The combination of HPLC with a multiwavelength ultraviolet (UV) detector (e.g., diode-array detector (DAD) [7–9]) offsets the disadvantage of lower resolution by allowing the acquisition of UV spectra, and produces systems with a discrimination number comparable to capillary GC. Additional advantages of HPLC are the excellent quantitative performance which is easily obtained for drugs which have proved "difficult" by capillary GC (e.g., underivatised phenobarbitone, propoxyphene and quinine), and the relatively simple sample preparation steps required for the analysis of biological specimens. The major obstacles concerning the incorporation of HPLC procedures into systematic analytical schemes for toxicology have been the variations in retention parameters, which have been obtained between different batches of column packing material, and changes which occur during the lifetime of a single column. In order to compensate for this variability, retention index (RI) or relative retention time (RRT) can be used as a method of correction. Retention indices are based on the relationship between a substance's retention time and those of a number of reference compounds (markers). The reference compounds are assigned RI values and the retention times of other substances are converted to RI values by linear interpolation between the nearest pair of reference markers. As an alternative, the retention time or capacity factor of a substance can be expressed as a proportion of that of a single reference compound. As these are measured relative to reference markers, they are independent of variations in absolute retention time, providing "corrected" retention parameters.

Previously published work has examined the effectiveness of using homologous hydrocarbon series [10–13], single or multiple drug reference standards [14,15] and most recently nitroalkanes [16,17], correct drug HPLC retention data. The development of a drug screening system published by Bogusz et al. [18,19] was particularly ambitious as it involved an approach, fundamentally based on a nitroalkane retention index scale, which could be applied to standardise data from any octadecyl or octyl reversed phase column, regardless of manufacturer.

Several conclusions can be drawn from the published literature: the use of a single type of column from one manufacturer produces the most reproducible results. Correction by means of RI improves reproducibility, but it is difficult to obtain linear RI scales based on a homologous series of compounds during gradient elution [20]; the most suitable HPLC mode for toxicology screening [21]. The most effective correction of HPLC retention parameters for drugs is obtained by the use of multiple drug standards, rather than non-drug compounds [14,18]. Thus, Bogusz et al. [18,19] found it necessary to incorporate a correction of their nitroalkane retention indices, by means of mixtures of basic and acidic/neutral drugs, in order to obtain close agreement of drug retention parameters, even between columns of different batches from a single manufacturer.

The most important factor in the effectiveness of a toxicological database is the resolving power (i.e. the number of compounds which can be separated) which is determined by the efficiency and selectivity of the column. Most of the previously published screening systems use an octadecylsilane (ODS) column which provides good separation of many compounds, however, the column packing material chosen for this work was a mixed alkyl/nitrile bonded phase silica Spherisorb S5OD/CN. This is available world-wide from a large, well-established manufacturer (Phase Separations, Clywd, UK). Previous experience of this material (unpublished observations) has indicated that it has good resistance to acidic aqueous mobile phases containing amine modifiers and shows a different selectivity from ODS and octyl bonded phase silicas.

The object of the present study was to develop a general HPLC drug screening system suitable for inclusion in an existing analytical scheme involving GC and TLC. Such a system is most powerful when combined with a diode-array UV detector or mass spectrometer. An important requirement of the system was high and long-term reproducibility of corrected retention parameters, both between columns and within the useful lifetime of a single column. This was considered a prerequisite for the establishment of a large database of compounds and associated metabolites, such as those compiled for capillary GC.

2. Experimental

2.1. Chromatographic equipment

Experiments were performed using a M480 high precision pump, column oven, Gina 500 autosampler and a UVD340S diode-array UV detector, all from Gynkotek (c/o HPLC Technologies, Macclesfield, UK), with a X-Act 4-channel degasser from Jour Research, (Onsala, Sweden). Four Spherisorb S5OD/CN 4.6×150 mm I.D. cartridge columns (Phase Separations, Clwyd, UK), protected by 10×4 mm guard columns of Spherisorb S5ODS2 were used for the studies. A saturation column was not used. Data acquisition was handled by a Gynkosoft software package running on an Elonex 560/I Pentium PC 60 MHz, 16 Mb RAM with the diode-array UV detector recording spectral data between 200 and 595 nm.

2.2. Materials

The 1.0 *M* triethylammonium phosphate buffer (pH 3.0) and nonylamine (98%) were supplied by Fluka (Gillingham, UK) and the HPLC-grade acetonitrile and HPLC-grade methanol were supplied by Rathburn (Walkerburn, UK).

The drugs used as standards in the investigation were obtained from various pharmaceutical companies or purchased from Sigma–Aldrich (Poole, UK). Lorazepam benzophenone (2-amino-2′,5-dichlorobenzophenone) was produced by acid hydrolysis of a methanolic solution of benzodiazepine with concentrated HCl, followed by evaporation to dryness and reconstitution with HPLC-grade methanol.

A mixture of each of the reference drug standards was made to a concentration of 0.1 mg/ml in methanol (HPLC-grade), except for ephedrine which was 0.25 mg/ml. The injection volume was 10 μl.

The various drug standards were dissolved in HPLC-grade methanol to a concentration of 1 mg/ml, and diluted prior to injection to a final concentration of 0.1 mg/ml with methanol. The injection volume was 10 μl.

2.3. Preliminary studies investigating the optimum method and conditions

An important parameter in gradient elution is the mobile phase composition and its pH [22]. Nonylamine was used as the amine modifier in the initial gradient elution studies as it is in several current in-house isocratic HPLC techniques, with a phosphate buffer and acetonitrile. The nonylamine was added (0.08%, v/v) to the KH_2PO_4 (0.2 *M*) and the pH was adjusted with *ortho*phosphoric acid. It was found, however, that the elution baseline contained various extraneous peaks caused by impurities in the nonylamine (Fig. 1A). This occurred with different batches of nonylamine and has also been reported by Bogusz et al. [18]. A commercially available amine modifier and pH buffer, triethylammonium phosphate, as suggested by these authors, was used in all further studies as it produced a reproducible baseline with fewer extraneous peaks and disturbances (Fig. 1B).

Initial studies also involved the use of both mobile phases adjusted to pH 3.0 but as this approaches the pH limit of most silica based columns (affecting column stability), the mobile phases were adjusted to a slightly higher value of pH 4.0 to minimise the possibility of column degradation. This produced an unacceptably high elution "ramp" of the chromatogram which was partly solved by using unadjusted acetonitrile (approximately pH 4.0) and unadjusted buffer (pH 3.0).

2.4. Final working method

A single step gradient elution was performed using a mixed mobile phase. Mobile phase A consisted of 700 ml 99.9% HPLC-grade acetonitrile together with 25 ml 1 *M* triethylammonium phosphate buffer (pH 3.0) diluted with 275 ml HPLC-grade water to give a final concentration of 25 m*M* buffer and 70% acetonitrile. The resulting mixture was not pH adjusted and the resultant pH, as measured by a pH meter, was pH 4.0. Mobile phase B consisted of 25 ml 1 *M* triethylammonium phosphate buffer diluted

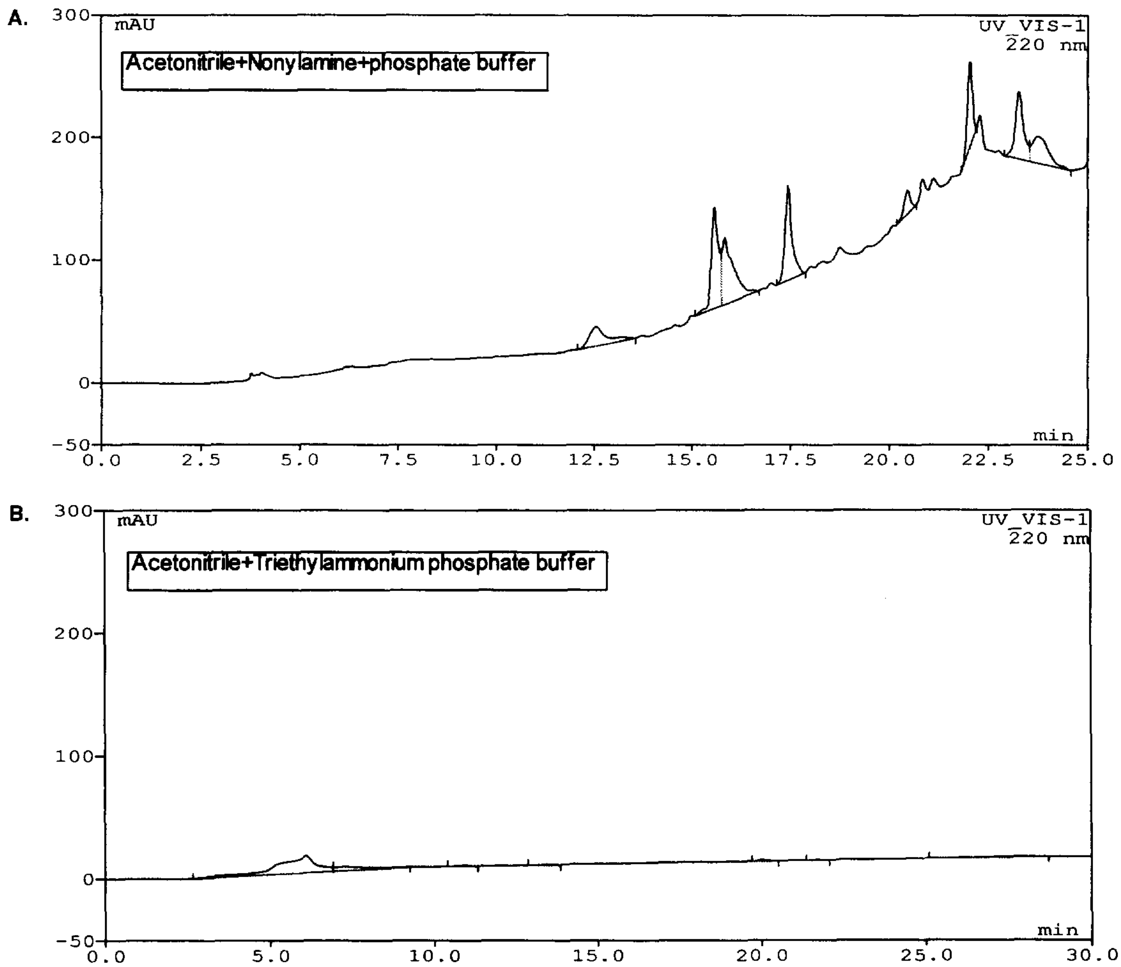


Fig. 1. (A) Chromatogram showing the effect on the 0–70% acetonitrile gradient baseline when using nonylamine with a phosphate buffer and acetonitrile, both adjusted to pH 4.0. (B) Chromatogram showing the effect on the baseline of a 0–70% acetonitrile gradient in 25 min when using unadjusted triethylammonium phosphate buffer (pH 3.0) and acetonitrile.

with 975 ml HPLC-grade water to give a final concentration of 25 m*M* buffer. This mixture was not pH adjusted and was therefore pH 3.0.

The elution conditions were achieved using a 2 ml/min flow-rate of 0–70% acetonitrile in 25 min then holding at 70% acetonitrile for 5 min. To purge the column and achieve an effective equilibration for the following analysis, the eluent was held at 70% acetonitrile for a further 5 min, then reduced from 70–0% acetonitrile over 5 min and finally held for 5 min. This gave a total run time of 45 min between injections, with data acquisition during the first 30 min (Fig. 2). The column temperature was maintained at 25°C.

2.5. Marker selection for RI calculations

Previously published HPLC systems for correcting retention data have generally used a series of homologous non-drug compounds as markers; however, during gradient elution these produce a non-linear retention scale. Such non-linearity would make it difficult to use an RI database to confidently predict the resolution of closely-eluting compounds,

Time (mins)	% Acetonitrile
0	0
25	70
30	70
35	*70*
40	*0*
45	*0*

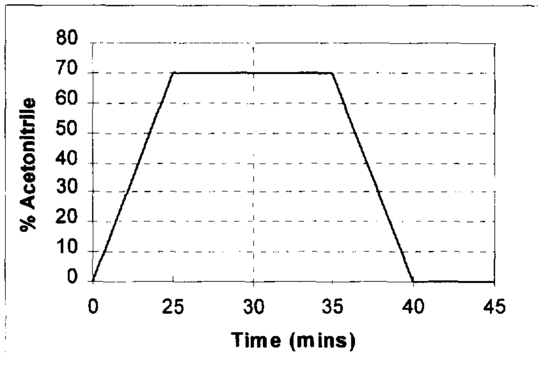


Fig. 2. Elution profile of the 0–70% acetonitrile and triethylammonium phosphate buffer gradient. Data acquisition occurred within the first 30 min followed by a 15 min equilibration period (shown in italics).

since a defined degree of resolution corresponds to different RI intervals at different points in the scale. This seriously limits the practical usefulness of a database, e.g., for selecting strategies for quantitative analysis. It was therefore decided to produce a linear retention scale by selecting marker drugs which elute at regular intervals and cover the expected elution range of the compounds to be analysed.

Drugs to be used as reference markers were selected based on certain characteristics such as stable retention times relative to the other markers, resistance to dissociation and conversion to other substances, and sufficient UV absorbance at the selected monitoring wavelength. The reference markers were chosen to produce a stable linear retention scale during the gradient elution. Initially a mixture was selected containing 5 basic drugs, each being assigned arbitrary RI values of 100, 200, 300, 400 and 500. In-order to investigate the possible need for a separate RI scale for acidic and neutral drugs, an additional mixture of 5 acidic/neutral drugs was selected (based on the same criteria) which provided a second linear RI scale. Fig. 3 shows the complete reference mix containing the reference markers for the basic and acidic/neutral RI scales.

2.6. Calculation of RRT and RI values

To study the reproducibility and effectiveness of the RRT and RI correction methods, the RIs and RRTs of 48 basic drugs and 30 acidic/neutral drugs (Table 1 and Table 2) were determined on four Spherisorb OD/CN columns from different batches.

The RRT of the basic drugs were calculated with respect to two different single markers; diphenhydramine and amiodarone. In order to study the effect of using a basic drug as a single marker for acidic and neutral drugs, the RRT of the acidic/neutral drugs were calculated with respect to diphenhydramine, amiodarone, demoxepam and lorazepam benzophenone.

$$\mathrm{RRT} = (t_R / t_1),$$

where t_R = retention time of toxicological substance (min); t_1 = retention time of single marker (min).

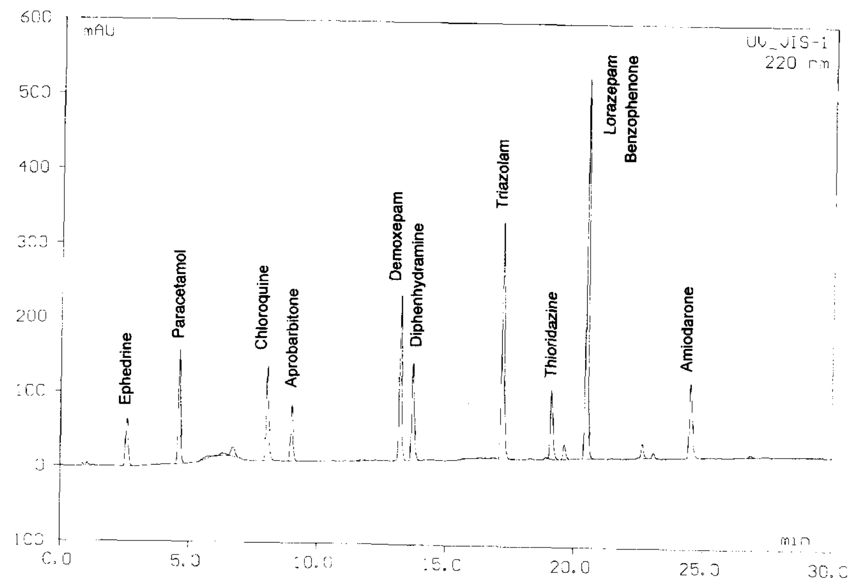
RI values were calculated for acidic and neutral drugs by interpolation between the acidic drug markers and for basic drugs by interpolation between the basic drug markers, using the formula stated below (after the position of the substance had been determined between two of the markers):

$$\mathrm{RI} = (t_R - t_1 / t_2 - t_1) \times 100 + \mathrm{RI}_1$$

where t_R = retention time of toxicological substance (min); t_1 = retention time of "first" marker (min); t_2 = retention time of "second" marker (min); RI_1 = RI value of "first" marker.

2.7. Extraction methods for biological specimens

Whole blood, serum, plasma and urine samples were extracted using in-house liquid–liquid extrac-



Basic RI Scale

Reference compound	Assigned RI Value
Ephedrine	100
Chloroquine	200
Diphenhydramine	300
Thioridazine	400
Amiodarone	500

Acidic/Neutral RI Scale

Reference compound	Assigned RI Value
Paracetamol	100
Aprobarbitone	200
Demoxepam	300
Triazolam	400
Lorazepam benzophenone	500

Fig. 3. Chromatogram to show the elution order of the reference mix containing the markers used to calculate the basic and acidic/neutral RI scales. A 0–70% acetonitrile gradient in 25 min, followed by a hold at 70% acetonitrile for 5 min, was used for the chromatographic conditions.

tion techniques associated with existing GC and HPLC systems. For basic drugs 200 μl 0.2 *M* Na_2CO_3 buffer was added to 500 μl of sample followed by 5 ml of 1-chlorobutane. After 3 min shaking and centrifugation at 1310 *g* for 3 min, the supernatant was transferred to a second tube and drugs were extracted into 100 μl 0.05 *M* H_2SO_4. 20 μl of the acid layer was transferred into a vial for injection. For acidic/neutral drug extractions 500 μl 0.05 *M* H_2SO_4 was added to 500 μl of sample with 5 ml chloroform added as the extractant followed by 3 min shaking and centrifugation at 1310 *g* for 3 min. The supernatant was filtered through chloroform-saturated filter paper, evaporated to dryness and reconstituted with 100 μl methanol prior to injection. The injection volume was 20 μl.

Table 1
The 48 basic drugs used to compare the RI and RRT correction methods

Amitriptyline	Dothiepin	Nortriptyline
Amphetamine	Doxepin	Orphenadrine
Benzoctamine	Fenfluramine	Oxprenolol
Benzoylecgonine	Fluvoxamine	Pentazocine
Brompheniramine	Labetolol	Pindolol
Caffeine	Lignocaine	Propranolol
Carbamazepine	Meclozine	Pseudoephedrine
Chlorpheniramine	Methadone	Quinine
Cimetidine	Metoclopramide	Theophylline
Clomipramine	Metoprolol	Timolol
Cocaine	Morphine	Tocainide
Codeine	Nicotine	Tranylcypromine
Desipramine	Nordothepin	Trazadone
Dextropropoxyphene	Nordoxepin (trans)	Trifluperazine
Dibenzepin	Norfenfluramine	Verapamil
Diltiazem	Norpseudoephedrine	Zopiclone

2.8. Calibration protocol

The reference mixture (containing the 10 drugs which produce the basic and acidic/neutral RI scales) is injected to calibrate the system on a daily basis, prior to the analysis of any standard compounds or biological extracts.

Table 2
The 30 acidic/neutral drugs used to determine the requirement for two separate RI scales and to compare the RI and RRT correction methods

Allobarbitone	Indomethacin	Nordiazepam
Butalbarbitone	Ketazolam	Oxazepam
Butobarbitone	Lorazepam	Oxazepam benzophenone
Chlordiazepoxide	Lormetazepam	Pentobarbitone
Clobazam	Medazepam	Phenobarbitone
Clonazepam	Methylbromazepam	Phenytoin
Diazepam	Methylnitrazepam	Prazepam
Diazoxide	Midazolam	Temazepam
Flunitrazepam	Nitrazepam	Trimethoprim
Flurazepam	Norclobazam	Warfarin

3. Results

3.1. The requirement for separate basic and acidic/neutral RI scales

The RI values for 30 acidic/neutral drugs were determined using two OD/CN columns from different batches of packing material. The statistical method of linear regression was used to assess the RI reproducibility between columns. RI values were calculated using both the basic and acidic/neutral drug reference scales. The results for the RI values determined using the basic RI scale gave an r^2 value of 0.9973. When the RI values for these drugs were determined using the RI scale based on acidic/neutral reference markers, the RI values gave an r^2 value of 0.9988.

The RI values of the 30 acidic/neutral drugs were also determined on a single OD/CN column at the beginning and end of a 10 month period of routine and development use. RI values based on the basic RI scale produced an r^2 value of 0.9964. The RI values based on the acidic/neutral RI scale gave an r^2 value of 0.9983. In particular, it was noticed that the 6 barbiturates exhibited poor RI reproducibility when determined using the basic RI scale (Table 3 and Table 4).

3.2. Comparison of RI and RRT correction methods

3.2.1. Column–column reproducibility

To study the reproducibility of both the RI and RRT correction methods between columns, the RI values and RRT values for 48 basic and 30 acidic/neutral drugs were calculated using four columns from different batches of Spherisorb S5OD/CN. The RI values for the basic and acidic/neutral drugs were

Table 3
The column to column reproducibility, and long-term (10 months) reproducibility of RI values for 6 barbiturates calculated using a basic RI scale based on basic reference drugs

Substance name	RI values			
	[a]Batch no. 94/344	Batch no. 95/123	Batch no. 95/123 August 1995	Batch no. 95/123 June 1996
Allobarbitone	189	199	199	189
Aprobarbitone	206	215	215	206
Butalbarbitone	235	246	246	237
Butobarbitone	232	242	242	233
Pentobarbitone	268	275	275	269
Phenobarbitone	221	230	230	219

[a] The batch no. refers to the batch reference number of the ODCN column packing material used in the study.

Table 4
The column to column reproducibility, and long-term (10 months) reproducibility of RI values for 6 barbiturates calculated using an acidic/neutral RI scale based on acidic/neutral reference drugs

Substance name	RI values			
	[a]Batch no. 94/344	Batch no. 95/123	Batch no. 95/123 August 1995	Batch no. 95/123 June 1996
Allobarbitone	177	180	180	177
Aprobarbitone	200	200	200	200
Butalbarbitone	238	242	242	237
Butobarbitone	233	236	236	232
Pentobarbitone	277	280	280	274
Phenobarbitone	219	220	220	216

[a] The batch no. refers to the batch reference number of the ODCN column packing material used in the study.

calculated using the basic RI scale and acidic/neutral RI scale; linear regression produced mean r^2 values of 0.99929 and 0.99914, respectively (Table 5 and Table 6). The worst RI reproducibility observed between two OD/CN columns gave a correlation coefficient of 0.999 and an r^2 value of 0.9991 (Fig. 4A). Overall, the reproducibility of the basic drugs produced a mean standard deviation of ±1.65 RI units and the acidic/neutral drugs produced a similar mean standard deviation of ±1.67 RI units. The RRT of the basic drugs were calculated relative to diphenhydramine and amiodarone, and the RRT of the acidic/neutral drugs were calculated relative to diphenhydramine, amiodarone, demoxepam and lorazepam benzophenone. All of the single markers produced very similar mean r^2 values, however RRT calculated relative to diphenhydramine gave the best reproducibility for basic and acidic/neutral drugs (mean r^2 values of 0.99974 and 0.99897, respectively); see Table 5 and Table 6.

3.2.2. Reproducibility over a period of time

To study the reproducibility with time of both the RI and RRT correction methods, the RI values and RRT values for 48 basic and 30 acidic/neutral drugs were calculated after a 10 month period using a single OD/CN column. RRT and RI values were calculated as for the column–column study, using

Table 5
The RRT and RI reproducibility of 48 basic drugs, between 4 columns from different batches of ODCN

	RRT single marker Diphenhydramine	RRT single marker Amiodarone	RI values
Mean correlation coefficient (r)	0.99987	0.99977	0.99965
Mean r^2 value	0.99974	0.99955	0.99929

Table 6
The RRT and RI reproducibility of 30 acidic/neutral drugs, between 4 columns from different batches of ODCN

	RRT single marker Diphenhydramine	RRT single marker Amiodarone	RRT single marker Demoxepam	RRT single marker Lorazepam benzophenone	RI values
Mean correlation coefficient (r)	0.99948	0.99948	0.99948	0.99948	0.99957
Mean r^2 value	0.99897	0.99896	0.99896	0.99896	0.99914

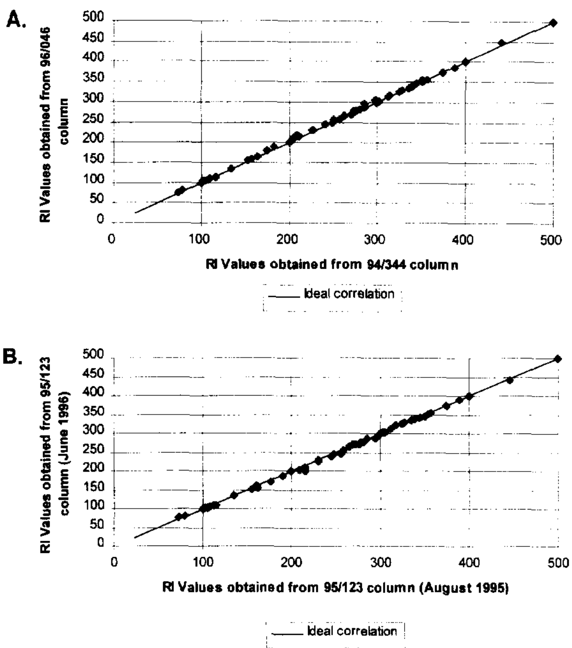


Fig. 4. (A) Scatter plot to show the worst column–column RI correlation. RI values of 48 basic drugs, based on the basic RI scale, were obtained using two new columns (correlation coefficient=0.9995, r^2=0.9991). (B) Scatter plot to show the RI correlation over a 10 month period (August 1995–June 1996). RI values of 48 basic drugs, based on the basic RI scale, were obtained using a single column (correlation coefficient=0.9993, r^2=0.9986, mean RI standard deviation=±2.13 RI units).

basic and acidic/neutral RI scales and 4 single marker reference compounds. The results (Table 7) for the RRT correction method of basic drugs produced an r^2 value of 0.9989, for both diphenhydramine and amiodarone. The RI correction method for the basic drugs produced a similar r^2 value of 0.9986 and an RI standard deviation of ±2.13 RI units (Fig. 4B). The reproducibility of the RRT values for the acidic/neutral drugs (Table 8) was very similar for all of the single markers, producing a mean r^2 value of 0.9984. Correction by the RI method (Table 8) produced a r^2 value of 0.9983 and a mean RI standard deviation of ±2.63 RI units. Due to an observed shift in the retention distance between the basic marker, chloroquine, and the acidic/neutral marker, aprobarbitone, the reproducibility of the RRT and RI values within the affected RI range of 100–300 (of both basic and acidic/neutral RI scales) was examined (Table 9 and Table 10). It was found that the RRT method of correction for the 29 affected basic drugs (Table 11) gave an r^2 value of 0.997, using both diphenhydramine and amiodarone as relative retention markers. The RI method for the basic drugs produced an r^2 value of 0.9964 (Table 9). For the 8 affected acidic/neutral drugs (Table 12), the RRT method produced an identical r^2 value of 0.9955, for each of the single markers. However, the RI method exhibited a slightly improved repro-

Table 7
The RRT and RI reproducibility of 48 basic drugs, after a period of 10 months using a single ODCN column

	RRT single marker Diphenhydramine	RRT single marker Amiodarone	RI values
Correlation coefficient (r)	0.99945	0.99946	0.99928
r^2 value	0.99891	0.99892	0.99856

Table 8
The RRT and RI reproducibility of 30 acidic/neutral drugs, after a period of 10 months using a single ODCN column

	RRT single marker Diphenhydramine	RRT single marker Amiodarone	RRT single marker Demoxepam	RRT single marker Lorazepam benzophenone	RI values
Correlation coefficient (r)	0.99922	0.99922	0.99921	0.99922	0.99914
r^2 value	0.99843	0.99841	0.99843	0.99843	0.99828

Table 9
The RRT and RI reproducibility of 29 basic drugs eluting in the RI range 100 to 300, after a period of 10 months using a single ODCN column

	RRT single marker Diphenhydramine	RRT single marker Amiodarone	RI values
Correlation coefficient (r)	0.99849	0.99852	0.99821
r^2 value	0.99698	0.99705	0.99643

Table 10
The RRT and RI reproducibility of 11 acidic/neutral drugs eluting in the RI range 100 to 300, after a period of 10 months using a single ODCN column

	RRT single marker Diphenhydramine	RRT single marker Amiodarone	RRT single marker Demoxepam	RRT single marker Lorazepam benzophenone	RI values
Correlation coefficient (r)	0.99775	0.99775	0.99775	0.99775	0.99831
r^2 value	0.99551	0.99551	0.99551	0.99551	0.99664

Table 11
The 29 basic drugs which elute in the RI range 100–300, used in the comparison of the RI and RRT correction methods

Amphetamine	Fenfluramine	Propranolol
Benzoctamine	Labetolol	Pseudoephedrine
Benzoylecgonine	Lignocaine	Quinine
Brompheniramine	Metoclopramide	Theophylline
Caffeine	Metoprolol	Timolol
Chloropheniramine	Morphine	Tocainide
Cimetidine	Norfenfluramine	Tranylcypromine
Cocaine	Oxxprenolol	Trazadone
Codeine	Pentazocine	Zopiclone
Dibenzepin	Pindolol	

Table 12
The 8 basic drugs which elute in the RI range 100–300, used in the comparison of the RI and RRT correction methods

Allobarbitone
Butalbarbitone
Butobarbitone
Chlordiazepoxide
Diazoxide
Pentobarbitone
Phenobarbitone
Trimethoprim

ducibility, producing an r^2 value of 0.9966 (Table 10).

3.3. Resolution and selectivity studies

3.3.1. Limit of resolution

Various resolution studies were performed using a single OD/CN column and a mixture of drugs which were selected to exhibit varying degrees of separation, dependent on their respective RI values. It was found that complete baseline separation occurred with a retention time difference of greater than 6 RI units in both the basic and acidic/neutral RI scales. For this study the "limit of resolution" was defined as the minimum RI difference (separation) between peaks which would allow the integration and spectral analysis of the individual peaks. An elution difference of 3 RI units gave sufficient separation for spectral analysis (allowing the acquisition of representative spectra for each of the eluted peaks) and integration; again this applied to both RI scales. A similar study was undertaken using the system previously published by Bogusz et al. [19], which is based on a 4 micron RP-18 ODS column. The limit of resolution (as defined above) was found to vary between 2–7 RI units (Bogusz RI units) due to the non-uniformity of the marker intervals.

3.3.2. Selectivity

Using the limit of resolution, it was possible to perform a selectivity comparison between ODS and OD/CN column packing material based on the (published) RI database of Bogusz et al. [19]. The coelutions and separations of 103 basic and 72 acidic/neutral drugs were predicted. A selection of these are shown to illustrate significant selectivity differences between the column packing materials, most notably verapamil, trimethoprim and amphetamine (Table 13 and Table 14).

3.4. Application of the system for biological specimens

3.4.1. Examples of the analysis of post-mortem blood and urine samples3

Fig. 5 and Fig. 6 are examples of a blood and urine basic drug screen based on the basic back

Table 13
A selection[a] of coelutions demonstrating the differences in selectivity between ODS and ODCN packing material for basic drugs

Substance name	Bogusz RI value	Toxicology RI value
Ephedrine	224	100
Atenalol	224	134
Theophylline	239	161
Amphetamine	241	112
Tranylcypromine	241	105
Tocainide	251	115
Verapamil	251	355
Caffeine	265	190
Chloroquine	265	200
Ketamine	294	189
Benzoylecgonine	295	214
Brompheniramine	355	272
Pentazocine	357	264
Trazadone	358	286
Chlormethiazole	369	263
Benzoctamine	370	281
Propranolol	370	279
Diphenhydramine	385	300
Paroxetine	385	330
Orphenadrine	416	326
Promazine	418	333
Nortriptyline	418	339
Fluoxetine	450	352
Trimipramine	451	356

[a] The complete comparison between the two packing materials was based on 103 basic drugs.

Table 14
A selection[a] of coelutions demonstrating the differences in selectivity between ODS and ODCN packing material for acidic/neutral drugs

Substance name	Bogusz RI value	Toxicology RI value
Trimethoprim	345	169
Diazoxide	345	217
Aprobarbitone	347	200
Chlorpropamide	356	299
Phenacetin	356	235
Phenobarbitone	357	220
Chlordiazepoxide	357	255
Midazolam	386	317
Demoxepam	388	300
Heptabarbitone	404	271
Hexabarbitone	404	266
Pentobarbitone	405	280
Glutethimide	430	299
Nitrazepam	430	333
Clonazepam	451	351
Triazolam	452	400
Nordiazepam	464	362
Glipizide	464	378
Temazepam	466	364

[a] The complete comparison between the two packing materials was based on 72 acidic/neutral drugs drugs.

extraction method. Caffeine (RI = 192) and the tricyclic antidepressant, dothiepin (RI = 336) and its metabolites (nordothiepin (RI = 325), nordothiepin

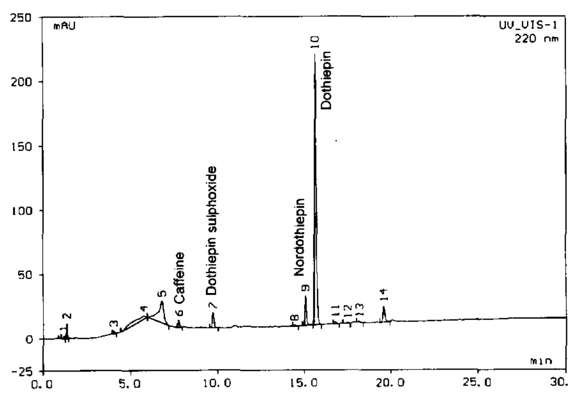


Fig. 5. Results of a drug screen performed on a post-mortem blood specimen using a 1-chlorobutane basic back extraction into 0.05 *M* H_2SO_4. Peaks were identified to be caffeine, dothiepin and metabolites by comparing the UV spectra with confirmation using the RI database. Few extraneous "system" and "matrix" peaks were observed. The elution conditions were based on a 0–70% acetonitrile gradient in 25 min followed by a hold at 70% acetonitrile for 5 min. Injection volume was 20 μl.

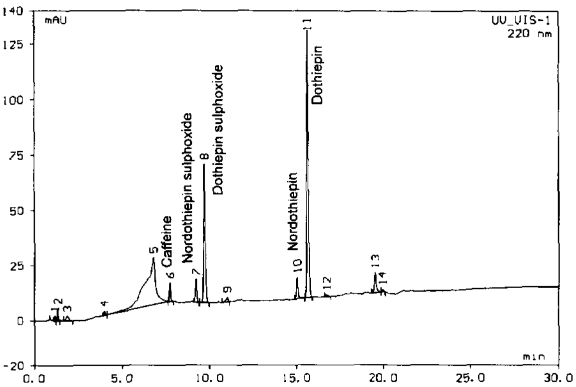


Fig. 6. Results of a drug screen performed on a post-mortem urine specimen using a 1-chlorobutane basic back extraction into 0.05 *M* H_2SO_4. Peaks were identified to be caffeine, dothiepin and metabolites by comparing the UV spectra with confirmation using the RI database. Few extraneous "system" and "matrix" peaks were observed. The elution conditions were based on a 0–70% acetonitrile gradient in 25 min followed by a hold at 70% acetonitrile for 5 min. Injection volume was 20 μl.

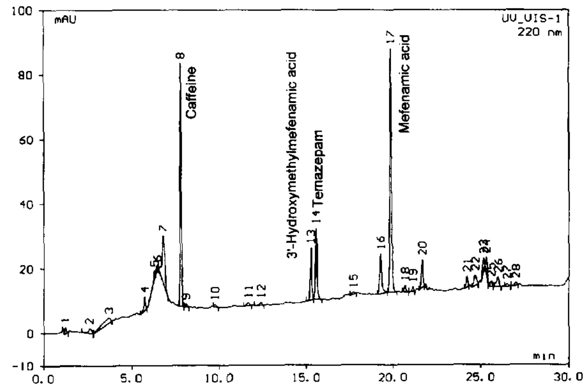


Fig. 7. Results of a drug screen performed on a post-mortem blood specimen using an acid/chloroform extraction method. Peaks were identified to be caffeine, temazepam, mefenamic acid and metabolites by comparing the UV spectra with confirmation using the RI database. A number of "matrix" peaks were observed after 21 min but did not interfere with the toxicologically relevant peaks. The elution conditions were based on a 0–70% acetonitrile gradient in 25 min followed by a hold at 70% acetonitrile for 5 min. Injection volume was 10 μl.

sulphoxide (RI=220) and dothiepin sulphoxide (RI=229)) were identified by RI and UV spectral matching. As previously mentioned, baseline disturbances due to the gradient elution conditions produce extraneous "system peaks". The most prominent of these [Peak 5; RI=174 (basic scale)] is observed at approximately 6.7 min, with a further extraneous peak at approximately 19 min [Peak 14 (Fig. 5) or Peak 13 (Fig. 6); RI=406 (basic scale)]. The other non-drug related peaks shown in both the urine and blood drug screens, are very small (2 mAU) and in these particular cases do not interfere with the toxicologically relevant peaks.

Fig. 7 shows the results of an acidic drug screen performed on a blood sample from a second case using the acid/chloroform extraction method. Caffeine (RI=192), temazepam (RI=360), mefenamic acid (RI=491) and its metabolite (3′-hydroxymethylmefenamic acid (RI=353)) were identified. Again the two "system peaks" can be observed (Peak 7; RI=150 (acidic/neutral scale) and Peak 16; RI=471 (acidic/neutral scale)), but there are numerous additional non-drug related peaks, presumably corresponding to components of the biological matrix, which are not observed when a back extraction method is used. The most prominent of these "matrix peaks" occur after 21 min (Peaks 20–28) and do not coelute/interfere with the toxicologically relevant peaks.

3.4.2. Influence of biological matrix on RI values

The effect of the biological matrix on the RI values of various basic and acidic drugs was studied using pure methanolic standards and extracted post-mortem blood and urine samples (not spiked). Table 15 shows that there was no significant effect on the calculated RI values. This reflects our experience of applying the system to approximately 100 clinical or forensic toxicology cases involving a wide range of drugs.

4. Discussion

4.1. The requirement for separate basic and acidic/neutral RI scales

Bogusz et al. [19] used separate sets of drugs as "secondary retention index standards" to correct 1-nitroalkane RI values of basic and acidic/neutral drugs, however, no experimental evidence was presented to show that this was necessary. On this OD/CN system, the reproducibility of 30 acidic/

Table 15
Comparison of retention index (RI) values of various acidic and basic drugs in pure methanolic solution and following extraction from post-mortem blood and urine specimens

Compound	Library RI value	RI_{pure} MeOH soln.[a]	RI_{blood} acid extract	RI_{blood} basic extract	RI_{urine} basic extract
Caffeine	192	194±0	192	192	192
Dothiepin sulphoxide	228	229±0	ND	229	229
Nordothiepin sulphoxide	221	N/A	ND	ND	220
Nordothiepin	325	323±1	ND	325	325
Dothiepin	336	335±1	ND	336	336
Temazepam	357	358±1	360	ND	ND
Mefenamic acid	488	490±1	491	ND	ND

[a] Mean±standard deviation calculated from three duplicate determinations.
N/A = Not available.
ND = Not detected.

neutral drug RI values determined when using only the basic drug reference scale was relatively poor (r^2 value = 0.9973). When the RI values for these drugs were determined using the RI scale based on acidic/neutral reference markers, the RI values showed a slight increase in correlation (r^2 = 0.9988). These results were obtained using two OD/CN columns from different batches of packing material, however, a similar difference in reproducibility was observed using a single column over a period of time. The RI values of the 30 acidic/neutral drugs, using a single OD/CN column over a 10 month period, were calculated using the basic RI scale and produced an 2 value of 0.9964. When the RI values were determined using the acidic/neutral RI scale, a greater degree of reproducibility was observed (r^2 value = 0.9983). This may be due to the acidic/neutral reference markers better reflecting the shift in retention behaviour of acidic/neutral drugs with time, than the basic reference markers. This was observed particularly with the barbiturates (weak acids), both column–column and over a period of time (Table 3 and Table 4). It can be seen that the reproducibility of the RI values for this group of drugs is increased by using the acidic/neutral RI scale. Thus, there appears to be an advantage in using separate RI scales for acidic/neutral and basic drugs, which is more significant for acidic and neutral compounds. Further comparisons with a larger number of drugs and inter-laboratory studies may determine whether the advantage is sufficient to justify the added complication of a dual RI scale.

4.2. Comparison of RI and RRT correction methods

4.2.1. Column–column reproducibility

It was found that of all the single markers used to calculate RRT, diphenhydramine produced the best RRT reproducibility for basic and acidic/neutral drugs between columns from different batches of OD/CN. This was interesting as it was thought that, as with the RI values, a greater degree of reproducibility would be achieved by calculating the RRT of acidic/neutral drugs relative to an acidic/neutral marker. The reproducibility of the RI correction method (Fig. 4A) was very similar to the RRT method for basic drugs (Table 5), however, for acidic/neutral drugs the RI method was slightly superior to the RRT method even when an acidic relative retention marker is used (Table 6). This suggests that a number of acidic/neutral reference markers are required across the elution gradient to correct for the shift in retention time between batches of stationary phase.

4.2.2. Reproducibility over a period of time

The results of a 10 month reproducibility study based on acidic/neutral and basic drugs using only one OD/CN column, also demonstrated that both the RI correction method and the RRT method showed excellent reproducibility, with all the single markers exhibiting a very similar reproducibility (Table 7 and Table 8). This was somewhat unexpected as it was thought that a number of reference markers, as opposed to a single marker, would correct for the possible loss of stationary phase and resultant shift in the retention time across the whole elution range with time.

During this 10 month study a gradual decrease in the retention distance between chloroquine and aprobarbitone was also observed; discovered due to the continual acquisition of the reference mix data. Despite this, it appeared that the RI values for both basic and acidic/neutral drugs over the whole elution range remained unaffected (Table 7 and Table 8), however, within the affected RI range of 100–300, the RI correction method exhibited better reproducibility for the acidic/neutral drugs than using the RRT correction method (Table 9 and Table 10). The reduction in the resolution of the two compounds seriously effected the practicality of having all the reference markers in one mix in-order to calculate the RI values within the 100–300 range of both scales. This reduction in retention of the column also suggested that the column was approaching the end of its life, and would not have been detected with the single marker RRT correction method. Hence, the reference mix can also be used as a "probe" for column deterioration.

Overall, although the results of these experiments show only a marginal advantage of RI over RRT correction methods, it is expected that further studies involving a larger number of drugs over a longer period of time would reveal the superior reproducibility of RI. It was for this reason, in addition to the application of the reference mix as a probe, that the RI correction method was chosen for further studies to investigate its effectiveness for drug screening.

4.3. Resolution and selectivity studies

4.3.1. Limit of resolution

The use of a linear retention scale enables the "limit of resolution" to be determined for the system. This is due to the retention time intervals between the markers remaining constant and therefore providing a constant time equivalent for a single RI unit throughout the whole elution range. Thus, 100 RI units corresponds to 5 min for the basic scale and 4 min for the acidic/neutral scale. It was found that, in the case of both the basic and acidic/neutral RI scales, an interval of 3 RI units between compounds produces sufficient separation of the peaks to allow integration and subsequent inspection of spectral data for each substance. Complete coelution may occur with substances that are less than 3 RI units apart, nevertheless, spectral data for the substances may be obtainable from the front and tail of the peak. However, the availability of spectral deconvolution or multi-component analysis software with some diode-array instruments (e.g., Varian), would enable identification of more closely eluting compounds. A direct comparison with the Bogusz et al. system [19] was difficult due to the inherent disadvantages of a non-linear retention scale. Experimental studies with this system indicated that the use of a packing material improved efficiency and provided a limit of resolution ranging from 2–7 nitroalkane RI units, depending on the marker intervals which ranged between 2.5 and 7 min per 100 RI units.

4.3.2. Selectivity

After determining the limit of resolution for the system it enabled the investigation of the possible selectivity advantages of using an OD/CN column compared to the more common ODS column. The results of a selectivity comparison between this OD/CN system and the Bogusz ODS system [19], suggested that the OD/CN column shows some significant differences in selectivity for both basic and acidic/neutral drugs (Table 13 and Table 14).

Coelution is a major factor in the effectiveness of a system for drug screening as possible coelutions must be taken into account in both clinical and forensic toxicology. This is of particular importance in forensic investigations where there is the possibility of many drugs being present, hence the probability of not being able to separate certain substances is vital in determining the cause of death. In particular, tricyclic anti-depressant drugs such as amitriptyline and imipramine have many associated metabolites, e.g., hydroxy and *N*-desmethyl metabolites, as do other drugs such as dextropropoxyphene and phenothiazines. As Bogusz et al. do not currently include many drug metabolite RI values [19], it is possible that further coelutions may occur using an ODS column. Therefore, an absolute comparison of OD/CN and ODS columns cannot be achieved and their application for clinical and forensic investigations cannot be completely assessed.

4.4. Application of the system for biological specimens

The application of this described system to the analysis of biological specimens is illustrated by the results obtained in blood and urine from two post-mortem cases. Studies of the effect of biological matrix on the RI values have shown that there is a negligible effect on the retention behaviour of the various drugs used in the investigation. This supports the applicability of the RI database values for identifying compounds in clinical and forensic investigations where urine, serum and blood specimens are usually analysed. Previous studies by Bogusz et al. have confirmed this [23]. The presence of system peaks observed in the examples does result in a slight loss in sensitivity, primarily in the region of approximately 6.7 min. However, as this takes the form of a baseline disturbance rather than an eluting compound, any drugs eluting in this region are observed as definite peaks on the "shoulder" of this disturbance, allowing peak integration and thus enabling compound identification by RI and UV spectral matching.

Matrix peaks, observed particularly in acid/chloroform extractions of a post-mortem blood and urine samples (e.g., Fig. 7), elute after 21 min and in this case did not interfere with the identification of the toxicologically relevant peaks. The majority of these matrix peaks consistently occur at approximately 25 min; since few drugs elute in this region these extraneous peaks should not interfere with the majority of investigations. Bogusz et al. has previously attempted to establish the identity of such matrix peaks, attributed to endogenous substances from the biological matrix, appearing in both basic and acidic extracts [23]. The authors identified 2-phenethylamine, tryptamine and indole in basic extracts of post-mortem blood and liver samples. The peak corresponding to indole appeared to be the predominant matrix peak observed in basic extracts. Additionally, in acidic extracts of serum, it was shown that a large matrix peak (over 100 mAU) eluted at approximately the same retention time as indole; this was identified to be 3-H-indazol-3-one. Based on these studies, UV spectral analysis was used to attempt to identify the same compounds in extracts of biological specimens using the present system. Only a few, small matrix peaks were observed in basic extractions of urine and blood, none of which were consistent with the identified and unidentified matrix peaks observed by Bogusz et al. Moreover, none of the matrix peaks observed in the acidic extraction of blood appeared to be 3-H-indazol-3-one.

4.5. Summary and future development

The results of this investigation have shown that a retention index scale based on multiple drug reference markers provides a very reproducible and predictable method of correction for retention data. The use of multiple drug markers as primary retention index standards in HPLC had not been previously applied to general drug screening and so far the results are very encouraging for both basic and acidic/neutral drugs. It has also been shown that there are potentially useful selectivity differences between ODS and OD/CN packing material. Using the described extraction methods for blood and urine, we have observed no significant effect of coextracted material on the calculated RI values of a wide range of basic, acidic and neutral drugs.

At present the RI database is being expanded and work is in progress to ascertain the effectiveness of this system as a routine method for drug screening. It is expected that the system will be incorporated into our current strategy for toxicological investigations and be utilised for both quantitative and qualitative analyses. Although diode-array UV detection is currently the most widely available information rich HPLC detection method, in the near future LC–MS will become more widely applied as the cost of the system is likely to fall. The RI database will still be applicable and remain an important adjunct to such systems.

Acknowledgments

The authors would like to thank Brian King of Phase Separations, Clywd, UK for supplying some of the columns used in this study, and Dr. Robin Braithwaite for his help during the preparation of this manuscript.

References

[1] M.E. Sharp, J. Anal. Toxicol., 11 (1987) 8–11.

[2] K. Pfleger, H.H. Maurer and A. Weber, Mass Spectral and GC Data of Drugs, Poisons, Pesticides, Pollutants and their Metabolites, 2nd ed., VCH Publishers, Weinheim, 1992.

[3] DFG-TIAFT, Gas Chromatographic Retention Indices of Toxicologically Relevant Substances on Packed or Capillary Columns with Dimethylsilicone Stationary Phases, Report XVIII of the DFG Commission for Clinical Toxicological Analysis, Special Issue of the TIAFT Bulletin, VCH Publishers, Weinheim, 1992.
[4] K. Wolff, M.J. Sanderson and A.W.M. Hay, Ann. Clin. Biochem., 27 (1990) 482–488.
[5] K. Wolff, M.J. Sanderson, A.W.M. Hay and I. Barnes, Ann. Clin. Biochem., 30 (1993) 163–168.
[6] DFG-TIAFT, Thin-Layer Chromatographic R_f Values of Toxicologically Relevant Substances on Standardised Systems, Report XVII of the DFG Commission for Clinical Toxicological Analysis, Special Issue of the TIAFT Bulletin, VCH Publishers, Weinheim, 1992.
[7] T. Fell and B. Clark, Eur. Spectrosc. News, 59 (1985) 13–16.
[8] A.F. Fell, H.P. Scott, R. Gill and A.C. Moffat, Chromatographia, 16 (1982) 69–77.
[9] A.F. Fell, H.P. Scott, R. Gill, B.J. Clark and D. Westerlund, J. Chromatogr., 286 (1984) 261–273.
[10] J.K. Baker and C-Y. Ma, J. Chromatogr., 169 (1979) 107–115.
[11] R.M. Smith, J. Chromatogr., 236 (1982) 313–320.
[12] R.M. Smith, T.G. Hurdley, R. Gill and A.C. Moffat, Chromatographia, 19 (1984) 401–406.
[13] R.M. Smith, T.G. Hurdley, R. Gill and A.C. Moffat, Chromatographia, 19 (1984) 407–410.
[14] R. Gill, A.C. Moffat, R.M. Smith and T.G. Hurdley, J. Chromatogr. Sci., 24 (1986) 153–159.
[15] E.M. Chan and S.C. Chan, J. Anal. Toxicol., 8 (1984) 173–176.
[16] M. Bogusz and R. Aderjan, J. Anal. Toxicol., 12 (1988) 67–71.
[17] M. Bogusz, G. Neidl-Fischer and R. Aderjan, J. Anal. Toxicol., 12 (1988) 325–329.
[18] M. Bogusz and M. Wu, J. Anal. Toxicol., 15 (1991) 188–197.
[19] M. Bogusz and M. Erkens, J. Chromatogr. A, 674 (1994) 97–126.
[20] R.M. Smith, R. Gill and M.D. Osselton, J. Anal. Toxicol., 13 (1989) 375–377.
[21] M. Bogusz, J. Anal. Toxicol., 15 (1991) 174–178.
[22] C.F. Buck and S.A. Tomellini, J. Chromatogr. Sci., 27 (1989) 166–171.
[23] M. Bogusz and M. Erkens, J. Anal. Toxicol., 19 (1995) 49–55.