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Journal of Chromatography A, 1000 (2003) 109–124
JOURNAL OF CHROMATOGRAPHY A
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Review

Application of comprehensive two-dimensional gas chromatography to drugs analysis in doping control

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Abstract

Comprehensive two-dimensional gas chromatography (GC×GC) now occupies a niche within the GC technology regime. The technique is undeniably unique in the manner in which the experiment is conducted, the way results are presented and the interpretive opportunities offered. For the 1000th volume of this journal it is appropriate to expand upon these features, and review the progress made in GC×GC to date. Firstly, brief general comment is made on multidimensional procedures, and to review key aspects of GC×GC. The use of the targeted multidimensional GC method allows absolute retentions in the second dimension of a GC×GC experiment to be estimated, and also offers a novel way to obtain enhanced response for resolved solutes. Then, to illustrate the utility of the technique, the application of GC×GC to the screening of drugs and their metabolites in biological fluids is described using prolintane metabolites in canine urine as an example, with samples taken at four time intervals after administration. This example illustrates the first application of GC×GC in the field of forensic toxicology, an area traditionally dominated by GC–MS. Most drug compounds were found to be retained on the 0.8-m second column for a greater time than the modulation period (3 s) used for initial analysis, under the conditions described. Hence a 0.4-m D2 BPX50 (50% phenyl methyl polysilphenylene) column was then used throughout, with most compounds retained less than 4 s. For the standard drug mixture, three overlapping drugs on the first dimension column (BPX5) were subsequently baseline resolved on the BPX50 column. For prolintane administration samples, the parent drug and metabolites could be effectively resolved from background matrix peaks. Likewise a 23-drug spike standard in horse urine blank gave acceptable resolution of the drugs from matrix peaks.

Keywords: Reviews; Drug analysis; Doping control; Gas chromatography, comprehensive two-dimensional; Prolintane metabolites

1. Introduction

Drug analysis is important to the pharmaceutical industry, in anti-doping control, illicit drug analysis and forensic sciences. Separation techniques are central to instrumental approaches for drug analyses [1], and cover the broad range of available chromatographic methods including gas chromatography–mass spectrometry (GCMS) (e.g. for the detection of non-steroidal anti-inflammatory drugs [2]), fast screening methods using high-performance liquid chromatography (HPLC) [3], capillary electrophoresis (CE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF-MS) (e.g. for enantiomer separation of

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0021-9673/02/$ – see front matter
doi:10.1016/S0021-9673(02)01998-2

racemorphan [4]), and may incorporate tandem mass spectrometry (MS–MS) approaches for enhanced sensitivity and/or identification [5]. This paper describes the application of the multidimensional method comprehensive two-dimensional gas chromatography (GC×GC) to the analysis of drugs and to the authors' knowledge represents the first report of the use of GC×GC in this field.

Ingested drugs are excreted either unchanged or as chemically modified metabolites. Drug metabolism is of importance in the pharmaceutical industry especially in determining the pharmacological activity, clinical efficacy, and toxicological profile of drug molecules. In forensic sciences, drug metabolism studies are also critical since the metabolite may be proof of ingestion or abuse of drugs. In doping control for example, metabolism of verapamil following oral and intravenous administration to the greyhound and oral administration to the horse [6] has been investigated. Prolintane metabolism and excretion in the greyhound [7] and the horse [8] has been followed by using GC–MS. Sensitive, simple and reproducible verapamil metabolism studies in various biological matrices using automated in-tube solid-phase microextraction (SPME) and HPLC–MS [9] has been reported. However, due to the fact that metabolites are often present at very low concentration in biological matrices that contain interferents such as salts, proteins and other small organic molecules, drug analysis in biological matrices is a challenge to chemical analysts. Isolation (extraction) of a given drug and its metabolites from the biological matrix, taking consideration of possible adsorption of the drugs onto matrix components, is a necessary precursor to the chromatographic separation, identification and quantification of the components. Thus, prior to analysis, extraction (e.g. solid-phase extraction) and often derivatisation (e.g. methylation or acetylation) is usually carried out to improve quantitative drug analysis. The general approach to use of SPE for analysis of the drugs of concern here has been reviewed [10]. Derivatisation is now a standard approach to improved GC analysis of polar molecules.

1.1. Multidimensional separation

Multidimensional separation methods involve two different separation dimensions, and are employed for a range of analytical reasons including removal of potentially interfering matrix components, or to improve the resolution of specific analytes. The hyphenation of two different sequentially coupled separation techniques (e.g. HPLC–GC, HPLC–CE) should normally guarantee that orthogonal separation mechanisms are achieved, thus ensuring that components are subjected to two largely independent separative displacements. The scope of hyphenated analytical techniques has been recently presented in Brinkman's text [11], whilst the theory and applications of multidimensional separations were thoroughly canvassed in the recent book by Mondello et al. [12]. It is also possible to use the same type of chromatographic method in a multidimensional experiment; for example, LC–LC has been used extensively for the analysis of drugs in matrices such as plasma, serum or urine. Eklund et al. [13] used a coupled RPLC and ion-exchange LC system in the determination of free concentration of sameridine, an anaesthetic and analgesic drug, in blood plasma. Another important requirement is that whenever two components are adequately resolved in any of the dimensions, they should remain resolved throughout the process [14]. Simmons and Snyder first attempted multidimensional GC (MDGC) in 1958 [15]. In this technique, heart-cut MDGC is carried out via a Deans switch (pressure balancing) whereby a small fraction of sample from a primary column is passed into a secondary column of greater selectivity. Heart-cut MDGC increases the resolution power for given zones or heart-cuts of a sample. The resultant peak capacity is the sum of the individual peak capacities of each column used. An application of MDGC to PCB specific-congener analysis demonstrated the required retention reproducibility on both the pre-column and analytical column that permitted reliable run-to-run analysis [16].

1.2. Comprehensive 2D GC

A significant development in the technique of MDGC arose in the early 1990s, when comprehensive two-dimensional gas chromatography (GC×GC) was proposed. A general review by Phillips and Beens summarises the development of GC×GC instrumentation and applications up to 1999 [17].

Since first proposed in 1991 [18], GC×GC has undergone a reasonably predictable (in hindsight) development. Years of relatively obscurity, with understandably little acceptance of the early unreliable modulation mechanism, were followed by investigations of new ways to achieve the GC×GC result. These studies specifically related to the modulation method. The schematic diagram of the GC× GC instrumental design (Fig. 1) assists in the following discussion. The first column (the pre-column or first dimension D1) is normally a non-polar or low polarity column of normal capillary column length and the separation mechanism is primarily via solute boiling point. The second column (the analytical column or second dimension D2) is a fast elution column (short length, narrow bore I.D. and thin film phase) with a more polar phase, hence the aim of the coupled system is to resolve closely eluting peaks of similar boiling point but of different

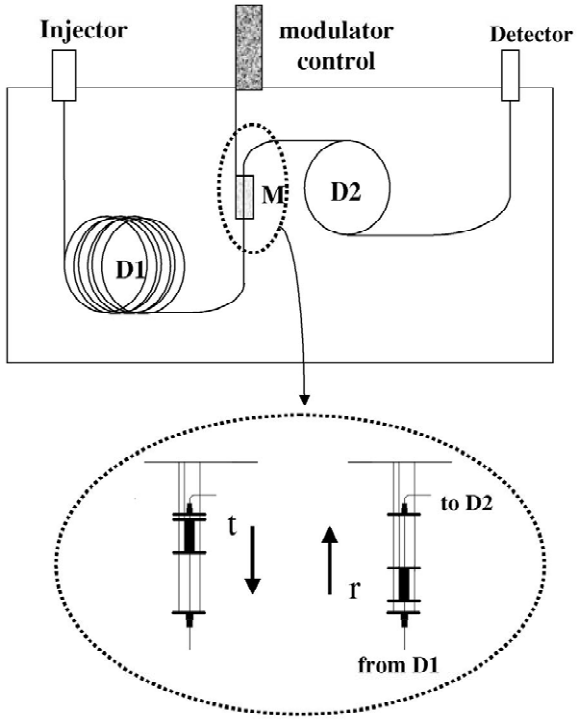

Fig. 1. Schematic diagram of a GC×GC system with the modulator M located between the first and second columns (D1 and D2). In this case, a moving cryogenic modulator is used, and the expanded region shows the cryotrap in its trapping (t) and release (r) positions.

polarity. The two columns must be ''orthogonal'' with respect to the separation mechanisms available to resolve components of the mixture, in order to provide maximum separation power, otherwise the purpose of performing GC×GC analysis may be defeated. Lee et al. [19] and others [20] reviewed the different modulation methods recently, however further modulators have been introduced since then. The three most widely used modulator types that provide mass conservation are the ''sweeper'' modulator [21], the longitudinally modulated cryogenic modulator (LMCS) [22], and the cryo-jet modulator [14,23]. They all serve the same purpose—to give the GC×GC result—but two different categories may be discussed. The first are those that provide complete sample transfer from the first to the second column (Seeley refers to this as having a duty cycle of 1.0 [24]), whilst the second may be considered to subsample only a part of the first column effluent. The latter systems are generally based on valve sampling methods, in contrast to the former which, in giving complete transfer of solute (i.e. conserve sample mass), will have directly coupled columns. In this case the modulator may be considered an on-column modulator.

1.3. Modulator performance

The modulator serves two functions: it must compress in time and space the solute band(s) as it (they) emerge from the first column, and secondly it must allow this compressed band to be quickly passed to the second column. According to Giddings' concepts of multidimensional chromatography, complete transfer of solute must be achieved, and to maximize multidimensional performance the separation that has been achieved in the first column must not be destroyed in this process [25]. Murphy et al. [26] determined that in order to maintain D1 separation, about four modulations or more are required for each ''peak width'' in D1. The extent of peak compression, the number of modulations per peak, and the narrowness of peaks generated at the end of the second column will contribute to the degree of peak response enhancement obtained in GC×GC [27]. The modulation of a peak thus effectively slices it into a series of pulses (which should reflect or mimic the input chromatographic

peak distribution, e.g. Gaussian), and the pulses possess a ''phase'' relationship with this distribution [28]. The actual pulse height sequence generated will depend on the phase and also any non-linear effects (such as peak overloading [29]). These, and related studies, serve to provide a fundamental basis to understanding the role of the modulation process. The cryogenic approach, developed by one of the collaborators in the present study, uses a moveable cryotrap (Fig. 1) which is supplied with liquid CO_2, and the two stage operation allows trapping of incoming solute when it is in position t, and rapid release of this trapped solute when it is moved to position r. Once trapped solute pulses into D2, the trap returns to position t, and the process repeats.

The GC×GC presentation is in the form of a 2D separation space (e.g. in contour plot format), with compounds spread throughout that space as a consequence of their retention property on each of the columns. GC conditions will affect the distribution of peaks in 2D space in a way which, whilst logical and in accordance with conventional GC principles, is more complex than arises in single column operation. The effect that chromatographic variables have on the GC×GC result is therefore a further consideration which must be taken into account for method development. This has been considered elsewhere [30,31]. The extension of GC×GC to fast operation, through the use of a short narrower bore (50 μm I.D.) second column than is usually used, has been demonstrated recently. Again, these studies extend the understanding of GC×GC performance and will support optimization studies for maximum separation performance.

The GC×GC technique, as any other, will only find a role if it offers an advantage to the analyst. This can only be achieved if, through testing the application boundaries and probing the subtleties of separations that GC×GC may provide, a greater qualitative and/or quantitative understanding of the chemical construct of samples is gained. The ability to use a 2D fingerprint is an immediate advantage that must be exploited to identify if sample comparison and general qualitative analysis is aided by such presentation. Since developments in chromatography are nothing if not a search for better separation in order to achieve the goal of materials' characterization, then the demonstration of enhanced separation in GC×GC must be widely applied to test the thesis that we can still discover new information from even ''old'', well studied applications. The applications base of GC×GC is expanding rapidly, and Table 1 lists an indicative range of these.

1.4. Cryogenic modulation modes

Two operational modes of the longitudinally modulated cryogenic system (LMCS), targeted MDGC (TMDGC) and the comprehensive GC (GC×GC) modes [32], are used in the work reported here (when the modulator is not ''activated'' by provision of CO_2, conventional one dimension GC analysis is obtained). The general concepts of TMDGC have been further demonstrated using a mixture of semi-volatile aromatic hydrocarbons [33]. For TMDGC, the trap is set to move at predetermined times to allow a selected peak, or set of peaks, to be cryogenically trapped and then fully remobilised on the second dimension column. The approach to solute trapping and remobilisation using the LMCS is described in Ref. [34]. If the trapped peaks have different physical properties that allow differentiation on the second dimension column, then overlapping ''trapped'' peaks can be separated. This method has the same conceptual approach to conventional MDGC, which employs heart-cutting. The significant difference is that there is no valve or flow switching involved. Thus in targeted mode, the selection of timing sequence is of importance. Prior to operation of the targeted mode, a normal GC analysis is performed to determine the required modulation event timetable so as to effectively cryotrap a complete peak or set of peaks before the trapped peak(s) is (are) released to the second dimension column. An additional modulation event is usually included just before cryotrapping the desired peak to remove extraneous peaks that might be collected in the trapping region (see later).

The modulator can also be operated using continuous oscillation, with a modulation period from 2 to 10 s, leading to the GC×GC technique. The sampling rate, i.e. the rate at which the modulator moves longitudinally through one cycle, depends on the frequency of sampling across each peak in the first dimension. In a study on comprehensive LC [26], Murphy stated that if the sampling is in-phase (in this case the modulator movement timing is such that it exactly captures the peak apex and equal portions

Table 1
Brief listing of selected applications of comprehensive two-dimensional gas chromatography

Application area	Analysis goal	Ref.
1. Petrochemical	Demonstration of tuned GC×GC analysis illustrating class separation of sample components	[36]
	Oil spill identification	[37]
	Identification of new geochemical marker compound	[38]
2. Forensic	Fire debris residues	[39]
3. Essential oils	Fingerprinting of different essential oil types	[40]
	GC×GC–TOF-MS of lavender	[41]
4. Environmental	PCBs enantiomer separation using chiral D1 column	[42]
	Organohalogen contaminants in complex mixtures	[43]
	Pesticides in vegetables	[44]
	Sterols	[35]
	Pesticides in plasma	[45]
5. Foods	Fatty acids	[46]
	Fatty acids in tuna and blue mussel	[47]
	Flavour components in wine	[48]
	Ginger volatiles using SPME	[49]
6. Chiral	Chiral first dimension column for essential oil analysis	[50]
	Chiral second dimension column for essential oil analysis	[51]
7. General	Volatiles from damaged grass	[52]
	Atmospheric organics in the urban atmosphere	[53]

of the peak to either side of the peak apex, giving a symmetric pulse distribution with one maximum), each peak in the first dimension should be sampled at least three times into the second dimension. However, if the sampling is maximally out-of-phase, each peak should be sampled at least four times to obtain high-fidelity separation. The peak in the first dimension would thus be ''sliced'' into several segments producing a series of three or more pulses into the second dimension column and it is important to note that the actual peak response of the GC×GC analysis is much greater than those obtained from normal GC analysis. Truong et al. [35] compared results such as peak heights, areas and reproducibility obtained from capillary gas chromatography operated in the normal mode with TMDGC and GC×GC. The frequency of pulsing should also not be so fast that components from successive second dimension analyses overlap. The data acquired are converted into matrix format on the basis of the modulator pulsing time to obtain a two-dimensional separation plane of retention times on the first dimension and second dimension columns.

Table 1 lists selected typical examples of applications of GC×GC, to illustrate the range of studies to which the technique has been applied. A more complete compilation has been recently summarised elsewhere [54], which essentially includes additional studies within the same general areas included in Table 1.

The aim of the present study is to develop general principles of GC×GC analysis for drug separation, and demonstrate the performance and typical separations which can be achieved. A comparison of data presentation in GC×GC, with conventional GC and GC–MS, is shown for the analysis of a complex mixture of drug standards and real samples of interest to the forensic toxicologist. Contour plots, which are obtained from GC×GC analyses of drugs and their metabolites, may be viewed as ''fingerprints'' for potential identification and rapid broad screening purposes.

2. Experimental

2.1. Chemicals and standards

The 27 drug standards investigated in this research, covering a range of primary, secondary and

tertiary amines, are shown in Fig. 2. Solutions (1 mg/ml) of each drug were prepared in water or methanol. Each standard, MSB 1 (containing drugs 1–10), MSB 2 (drugs 11–18) and MSB 3 (drugs 19–26) (code MSB refers to mass spectrometry basic drug standard), was then prepared by adding 300 μl

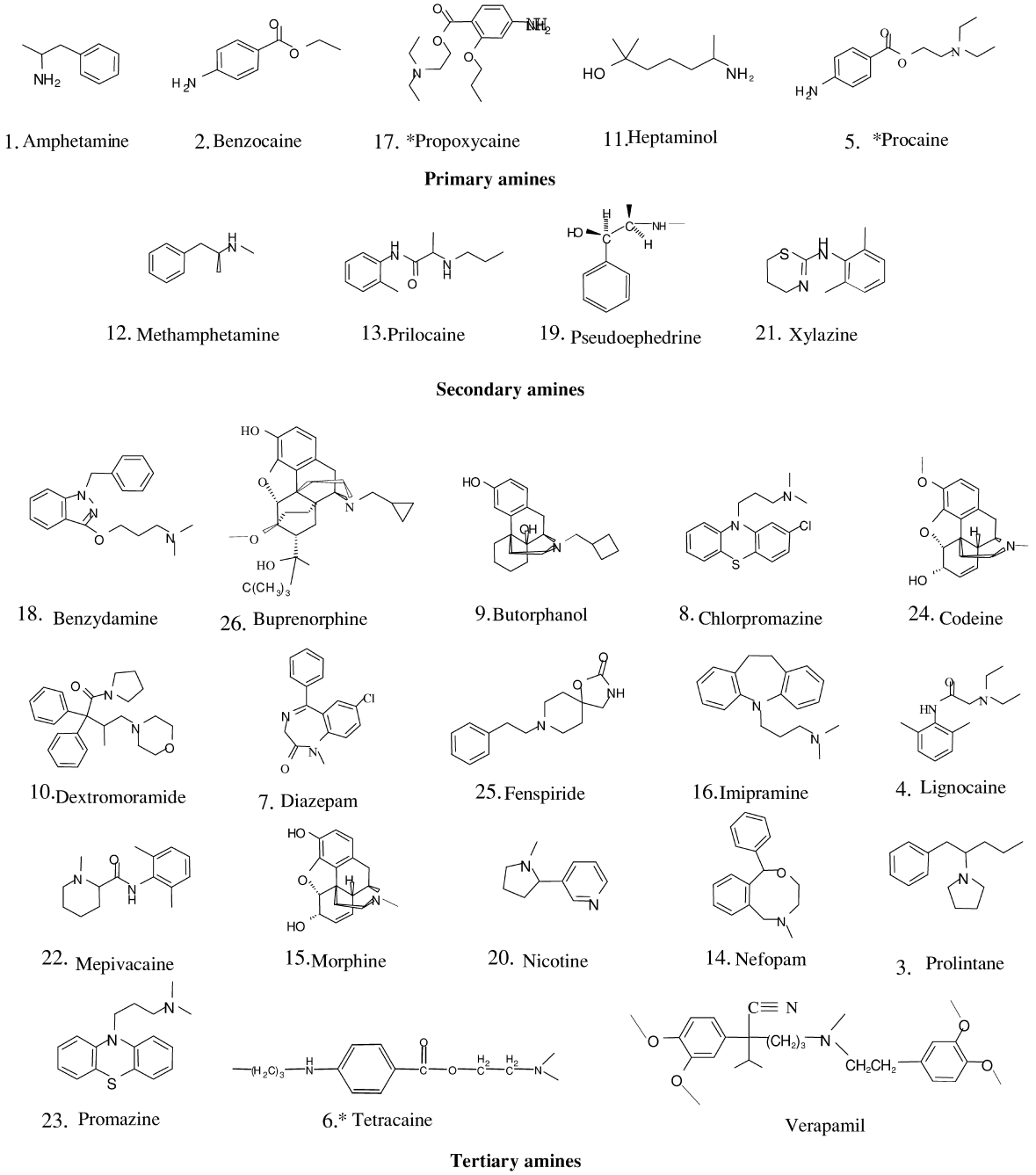

Fig. 2. Chemical structures of drugs in standards MSB 1, MSB 2, and MSB 3, plus verapamil.

of each solution to a 10-ml volumetric flask and made up to the mark with ethanol, giving a concentration of each standard of 30 mg/l. A drug standard mixture containing equal volumes of each of the MSB 1, 2 and 3 standards was also prepared. A stock solution of verapamil standard was prepared by dissolving 1 mg of powdered verapamil in 1 ml of water (i.e. 1000 mg/l), dispensed using a digital piston pipette. The five-figure balance was calibrated and certified (ISO 17025). A series of calibration standards of 2-, 5-, 8-, 10- and 20-mg/l concentration was prepared from the stock solution. This was repeated with aqueous prolintane.

Horse blank urine samples for the study of drugs in background matrices were spiked with 10 μl of the combined MSB standards described above. Both spiked blank urine and post-administration urine samples were extracted and derivatised as described below.

2.2. Pre-treatment of urine samples

Naturally voided urine samples from a greyhound that was orally administered 10 mg of prolintane hydrochloride (Catovit tablets, Boehringer Ingelheim, NSW, Australia) were collected after 0–1, 2.3, 5.7 and 24 h. Aliquots of 3 ml of each sample were transferred into a labelled sample tube followed by an addition of 4.5 ml phosphate buffer. The pH of the aliquots were adjusted to 6.8 using 1.0 *M* potassium hydroxide or 6.4% hydrochloric acid. A 50-μl sample of beta-glucuronidase enzyme was added to free the drugs that were conjugated as their glucuronides. At this stage, the urine samples were placed in an oven at 50 °C for 2 h. When the urine samples were cooled to room temperature, the pH was adjusted to 6.3 and centrifuged for 10 min at 3000 rpm.

2.3. Solid-phase extraction

The urine samples that were treated as described in Section 2.2 were extracted by SPE on mixed-bed Bond-Elut Certify Cartridges (100 mg bed mass; C_8/strong cation-exchange phase; Varian, Harbor City, CA). The SPE cartridge was conditioned with methanol (2 ml), water (2 ml) and phosphate buffer (0.1 *M*, pH 6, 2 ml). Each urine sample was then allowed to flow through the cartridge and the cartridge was dried and washed serially with acetic acid (1 *M*, 1 ml), chloroform:acetone (4:1, 2 ml), and methanol (6 ml). The retained material was then eluted with ethyl acetate containing 2% ammonia (2 ml). The eluate was evaporated to dryness at 60 °C under nitrogen and then reconstituted in ethyl acetate (100 μl).

2.4. Derivatisation

Based upon the polar nature of the drugs, acetylation of the amino group of the drug molecules with pyridine-acetic anhydride was used to produce more thermally stable derivatives and facilitate GC analysis. Urine sample extracts prepared in Section 2.3 were derivatised by acetylation with pyridine-acetic anhydride (2:1, 100 μl) in screw-capped culture tubes at 80 °C for 20 min. Excess reagent was destroyed with methanol (1 ml) and evaporated at room temperature under a stream of nitrogen. The residues were then washed with sodium tetraborate (1 ml) and extracted into dichloromethane (1 ml). The organic phase was dried over sodium sulfate and evaporated to dryness under a stream of nitrogen at 20 °C. The residues were reconstituted in ethyl acetate (100 μl).

2.5. Instrumental

GC–MS analyses were carried out using an Agilent model 6890 GC system (Agilent Technologies, Burwood, Australia) fitted with a model 6783 injector and 5973 mass selective detector. The column used was a HP-5MS (cross-linked 5% phenyl-methyl siloxane) with dimensions 30 m×0.25 mm I.D.× 0.25 μm film thickness.

GC×GC analysis was performed on an Agilent model 6890 GC system with a 6890 injector, which was retrofitted with a longitudinally modulated cryogenic system (LMCS; Chromatography Concepts, Doncaster, Australia) and FID detector. The column set used incorporated a primary column of dimensions 30 m×0.25 mm I.D.×0.25 μm film thickness BPX5 phase serially coupled with a second column of dimensions 0.4 m×0.1 mm I.D.×0.2 μm film thickness BPX50 phase. A D2 column length of 0.8 m was also used in preliminary experiments. The

modulation frequency, FID sampling rate and nominal trap temperature were 3 s, 50 Hz and 0 °C, respectively, unless otherwise stated.

All analyses were carried out in splitless injection mode with a purge on time of 1 min. The temperature programmes used for the analysis were as follows, unless otherwise stated. For the preliminary analysis of drug standards, the oven temperature was increased from 100 to 280 °C at a rate of 3 °C/min, holding at 280 °C for 10 min. This temperature programme was used for both the GC–MS and GC×GC analyses. For the analysis of real samples, the oven temperature was increased from 55 to 180 °C at a rate of 10 °C/min followed by 3 °C/min from 180 to 280 °C, then holding at 280 °C for 10 min. This is the same for both GC–MS and GC×GC analyses. Standards were also analysed under these conditions to provide comparative retention data.

3. Results and discussion

3.1. GC×GC analysis of drugs standards

Fig. 3A illustrates the GC trace of the combined drug standards mixture. Whilst it appears that all the peaks are well resolved, some component overlaps arise (for the peaks marked A and B). Fig. 3B shows the peak pulse presentation arising from the modulated GC×GC analysis; qualitatively this is very similar to the outline of the peaks in the normal GC trace in Fig. 3A. Fig. 3C is a contour plot chromatogram presentation of Fig. 3B. The peak indicated as A in Fig. 3A, apparently one single peak in normal GC, now obviously comprises three well resolved components (solutes 3, 11 and 19) under the GC× GC analysis procedure (Fig. 3C). Since the second dimension column has a separation mechanism of polarity and components 3 (secondary amine), 11 (primary amine) and 19 (tertiary amine) will have different polarity, they now have different second dimension retention times and hence are resolved when remobilized in the second dimension column. This may be regarded as a demonstration of the classic separation advantage of GC×GC. A small peak cluster appears just greater than noise level (baseline response) in Fig. 3A, at ~50 min. In Fig. 3C, these are readily recognized as a group of peaks at an apparent $^{2}t_{R}$ value of 0.8 s in the 2D space, well resolved from the drug components 7, 8 and 25.

Fig. 4 illustrates the comparison of the TMDGC method with the normal GC method, using the same experimental set-up as used in Fig. 3 and described in Fig. 1, in analysing the drug standard MSB 2. Fig. 4A is the normal GC trace of the standard, whilst Fig. 4B is the GC trace obtained from the targeted mode of analysis. The essence of this procedure is that complete peaks may be trapped by holding the cryotrap in its trapping position, then the collected volatile components are remobilized by moving the trap to the release position. This operation may be performed any number of times during the chromatographic analysis, and is controlled by using the event output signal from the software control to instruct the modulator to move at a predetermined time. Since the D2 column is short, with fast elution of the peaks, peak response height is increased. Thus peak 16 (imipramine) gave a peak height increase from 26 pA (normal GC) to 360 pA (TMDGC), and in general in this instance responses of some 15-fold increase are noted. Unless the same detector acquisition rate is used, it is not possible to directly correlate signal increase with sensitivity increase, since detector noise varies with the square root of the acquisition rate. There is essentially no difference in peak area. For peaks that are collected together in the one trapping event, it is still possible to achieve their resolution provided D2 gives sufficient difference in selectivity for these. Clearly D2 is a different stationary phase to D1, and for the same reasoning as choosing column orthogonality for the GC×GC experiment, it will be possible to resolve components using TMDGC. In this example, the CO_2 supply is commenced at 23 min, so all subsequent components (peaks 13–18) may be targeted according to the cryotrap movement event timetable. Components 11 and 12 were not cryotrapped in this case, and so their responses are not enhanced. Fig. 4A shows that methamphetamine (peak 12) has a tailing peak. This can reduce quantitative measurement accuracy of the component, however by fully collecting the peak and rapidly eluting it on D2, the peak symmetry improves and area and height measurement should be more precise.

Fig. 4B also presents some smaller peak responses

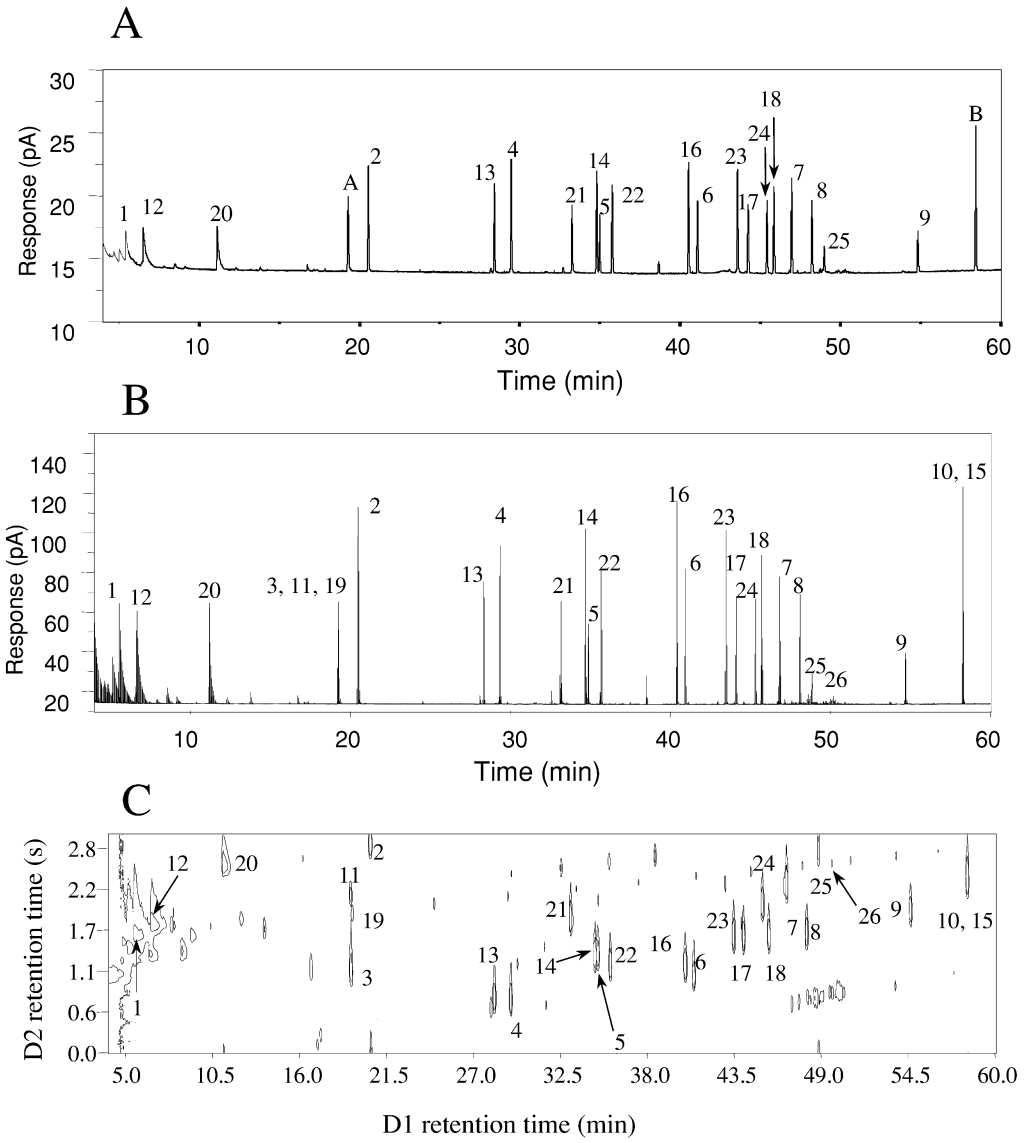

Fig. 3. Analysis of composite standard MSB 1+2+3. For conditions of analysis, refer to Experimental section. For identity of peaks, refer to Fig. 2. (A) Normal GC analysis; (B) pulsed GC×GC analysis; (C) GC×GC contour plot.

labeled 14a, 15a and 16a. These arise from the cryotrapping of small peaks subsequent to peaks 13, 14 and 18, respectively, and may be explained for peak 14a as follows. Since the cryotrap fluid remains on for the duration of the analysis, once peak 13 is released into the second column, any minor components that elute from D1 will be trapped in the cryotrap. As these accumulate in the trap, they potentially could interfere with the major component 14 when it is collected and released. So just prior to 14 entering the trap, we release any of these interfering peaks into D2 (they give the 14a response) then collect 14 without interference from these components. Likewise, the small peaks at ~50 min are all collected and released at 15a before peak 15 is collected and released.

The targeted mode offers a second opportunity for validation of GC×GC results, especially absolute

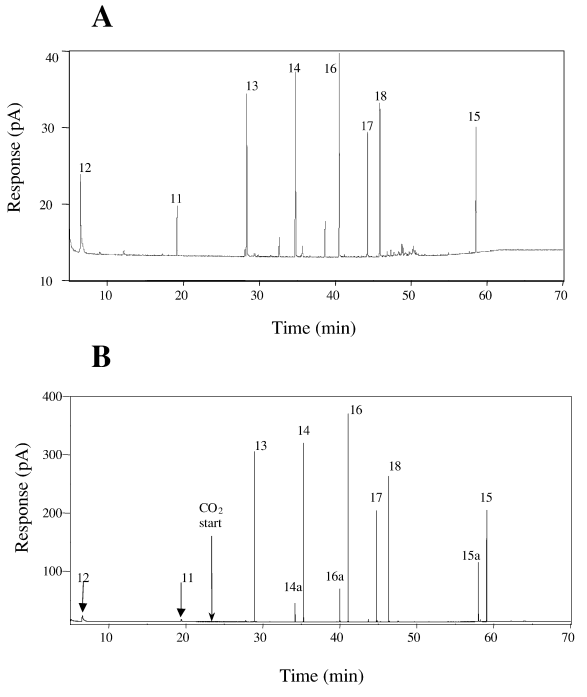

Fig. 4. Normal GC (A) and targeted multidimensional analysis (TMDGC; B) of drug standard MSB 2 using the same conditions as in Fig. 3. In (B), cryotrapping of components 13, 14, 15, 16, 17 and 18 only was conducted; 14a, 15a and 16a correspond to extraneous peaks cryotrapped before collecting the desired targeted peaks (refer to text). Peaks 11 and 12 are not cryotrapped (since CO_2 is commenced at 23 min, after elution of component 11), thus the intensity of the responses is small compared to the targeted peaks.

retention times on D2. The presentation of GC×GC data in the form of a 2D plot does not give any direct indication of elution times on D2. For instance there is no guarantee that a solute will elute from D2 within the modulation period of the experiment. If it elutes with greater retention time, then we have a situation where ''wrap-around'' arises. Various methods may be used to predict the absolute D2 time; comparison of peak positions when using different modulation periods, or alternatively peak width comparisons, may suggest that a peak experiences ''wrap-around'' (note that this will not necessarily reveal whether the solute undergoes more than one wrap-around). Hence, peak 11 in Fig. 3C is plotted at a D2 time of 2.1 s, but its actual D2 time may be 2.1, or 5.1 or 8.1 s, etc. (incremented by the modulation period of 3 s). By performing a targeted analysis, with collection of the full peak and having a single well-defined release time into D2, the absolute retention time of the solute on D2 (^2t_R) may be found. Since the solute is retained in the trap slightly longer than it is under the GC×GC experiment (where the trap/release process occurs during elution of the solute from D1), then the oven temperature advances a few degrees Celsius above that when the solute is analysed by the GC×GC mode. Hence the TMDGC retention time should be slightly less than that estimated from the GC×GC result, and the degree to which the TMDGC time is less than the GC×GC time depends on how long the solute has been held in the cryotrap above the elution temperature of the solute in the GC×GC mode. There will be no ambiguity in the ^2t_R value in the TMDGC mode. Table 2 presents a correlation of data for two different BPX50 D2 column lengths—0.8 and 0.4 m. It was apparent that under the conditions chosen, the 0.8 m column gave excessive wrap-around at 3 s modulation, with some components having in excess of 7 s retention. Shortening the column to 0.4 m still gave one wrap-around for some components, but this was deemed acceptable for the present study. The polar nature of the drug compounds clearly contributes to the longer retention on the D2 column.

Precision and accuracy of retention in the 2D plane are important for component identification, and thus the reproducibility of the GC×GC analysis must be evaluated. An overlay of contour plots of six consecutive GC×GC analyses for each of three different days (18 chromatograms) for mixture MSB 3 (not shown here) gave good correspondence and consistency of peak positions. The RSD of total retention time for the codeine component in these analyses (the time of the maximum pulse peak in GC×GC is used) and the codeine ^2t_R times for the same 18 runs above demonstrates that system reproducibility both between runs on 1 day (~0.1%) and between days (~0.05%) is excellent for total time, and is less than 1% for the D2 time, as shown in Table 3.

3.2. Calibration and detection limits

Calibration curves of two selected drugs, prolintane and verapamil, were plotted and the R^2 values are listed in Table 4. TMDGC analysis produced the

Table 2
Comparison of targeted analysis (TMDGC) and GC×GC D2 retention times on 0.8 and 0.4 m BPX50 D2 columns

Drug	0.8 m BPX50 D2 column		0.4 m BPX50 D2 column	
	^2t_R (TMDGC)[a]	^2t_R (GC×GC)[b]	^2t_R (TMDGC)	^2t_R (GC×GC)
Methamphetamine	2.34	2.50	1.68	1.78
Heptaminol	2.40	2.80	1.74	2.19
Prilocaine	5.16	5.95	3.18	3.75
Nefopam	6.00	7.10	3.54	4.35
Imipramine	5.52	6.70	3.36	4.20
Propoxycaine	5.46	7.63	3.60	4.61
Benzydamine	6.30	7.55	3.60	4.61
Morphine	7.14	8.83	4.14	5.41

[a] Times given in seconds.
[b] Times for GC×GC are derived from the closest match to the targeted time, TMDGC.

Table 3
List of retention times for component 24 (codeine) for six consecutive GC×GC analyses per day conducted over 3 days

	Day 1		Day 2		Day 3		Between days		
	^1t_R (min)	^2t_R (s)	^1t_R (min)	^2t_R (s)	^1t_R (min)	^2t_R (s)			
Run 1	45.4948	4.830	45.4892	4.821	45.4313	4.821			
Run 2	45.4000	4.833	45.5502	4.807	45.4949	4.918			
Run 3	45.4623	4.826	45.5354	4.798	45.4866	4.834		^1t_R (min)	
Run 4	45.5488	4.817	45.4500	4.781	45.4824	4.825	SD		RSD
Run 5	45.5396	4.812	45.5089	4.796	45.4792	4.829	0.0227		0.05%
Run 6	45.5296	4.787	45.5005	4.783	45.4000	4.812			
Average	45.4959	4.818	45.5057	4.798	45.4624	4.840		^2t_R (s)	
SD	0.0569	0.0170	0.0354	0.0150	0.0379	0.0390	SD		RSD
RSD	0.13%	0.35%	0.08%	0.31%	0.08%	0.81%	0.021		0.44%

The retention time of the largest pulsed peak is reported here.

most linear calibration curves for peak area data (0.9947 and 0.9978 for prolintane and verapamil, respectively), however all modes gave good calibrations. Note that for peak heights, because GC×GC gives a pulsed peak profile we can either sum up the total height of all pulses for a given compound, or we can choose the height of the most intense pulse. The latter will give greater uncertainty due to the effect of modulation phase [27], however it still gave acceptable results here. The detection limits for GC, GC×GC and TMDGC analysis were determined using the height response against concentration regression line. It was found that the detection limits were 0.1, 0.04 and 0.01 mg/l, respectively, thus TMDGC analysis gives the lowest detection limit, as anticipated.

Table 4
R-squared values of peak area calibration curves obtained for GC, targeted MDGC and GC×GC analysis for prolintane and verapamil calibration standards of 2, 5, 8, 10 and 20 mg/l

Drug	Calibration R^2 values (peak area)			Calibration R^2 values (peak height)[a]		
	GC	TMDGC	GC×GC	GC	TMDGC	GC×GC
Prolintane	0.9939	0.9947	0.9876	0.9925	0.9953	0.9863
Verapamil	0.9959	0.9978	0.9008	0.9846	0.9636	0.8895

[a] For GC×GC, the height of the largest peak pulse is used.

3.3. Application to real samples

Fig. 5 presents a contour plot of a horse urine background matrix sample spiked with the composite drug standard mixture. This study illustrates that the selected drugs are for the most part well resolved from the background matrix components. Under the temperature program conditions used here, procaine (5) and nefopam (14) are not resolved, whereas they were for the conditions used for Fig. 3. Since they have essentially the same ^2t_R values, if they are unresolved on D1 they will give an unresolved peak contour in the 2D plane. A homologous series of peaks observed in Fig. 5, with some members marked as S, corresponds to silanes, possibly derived from the sample preparation process where silanised sample tubes are used during the derivatisation step. Whilst the background matrices of urine may be different between horses there is every likelihood that the separation of drugs from matrix found here will be a general observation, with the location of the drugs in the 2D plane a useful fingerprint for screening or identification purposes.

A study of prolintane metabolites in the greyhound was undertaken, with urine samples taken at 0–1, 2.3, 5.7 and 24 h after oral administration (refer to Experimental section). GC–MS, GC and GC×GC analyses were conducted on the extracted samples. Fig. 6 illustrates selected metabolites that have previously been identified in dog urine [7] where I is the parent prolintane. Fig. 7 contrasts the GC–MS, GC and GC×GC chromatograms for the 0–1-h extract, and so allows an appreciation of data presentation for each method. The matrix, similar to that shown in Fig. 5, comprises a multitude of components that potentially may interfere in the single column GC analysis. Components of interest are more readily recognized in the GC×GC 2D space. Since the authentic metabolite compounds are not available as separate or pure compounds, their positions are inferred by comparison with the peak retentions in the most concentrated sample (that taken at 2.3 h). The GC×GC contour plot is drawn at a response level of 18 pA, and so not all minor peaks are shown. The most relevant point is that the latter plot permits ready identification of the metabolites, once their 2D position is established, in the other samples. Thus a masked 2D plot may be used as a screening tool where the appearance of peaks in the correct 2D windows would provide evidence of the presence of the drug metabolites. This is not dissimilar to the approach used in GC–MS analysis for these metabolites, where for example selected ion monitoring is performed with chosen ions within given retention windows. Fig. 8 shows the four GC×GC plots for the series of extracts. Again, the contour plotting level varies according to the intensities of the major metabolites for convenience of

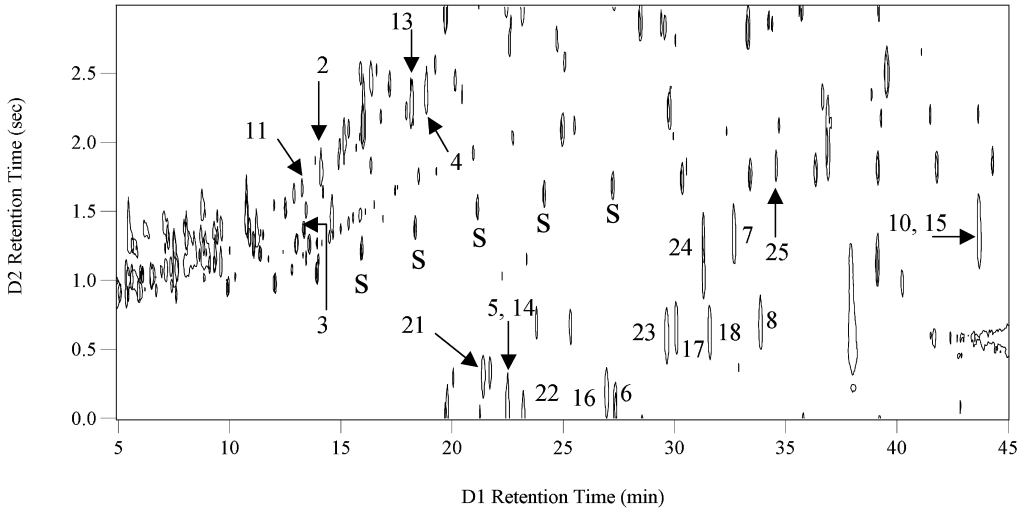

Fig. 5. Contour plot of a urine matrix spiked with drug standards mixture MSB 1+2+3, using the same conditions of analysis as Fig. 3.

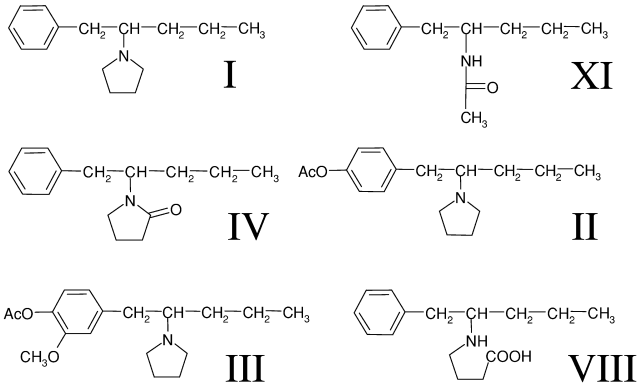

Fig. 6. Structures of various prolintane metabolites (or their acetate derivative) compared with the parent drug. The metabolite designations follow Ref. [7].

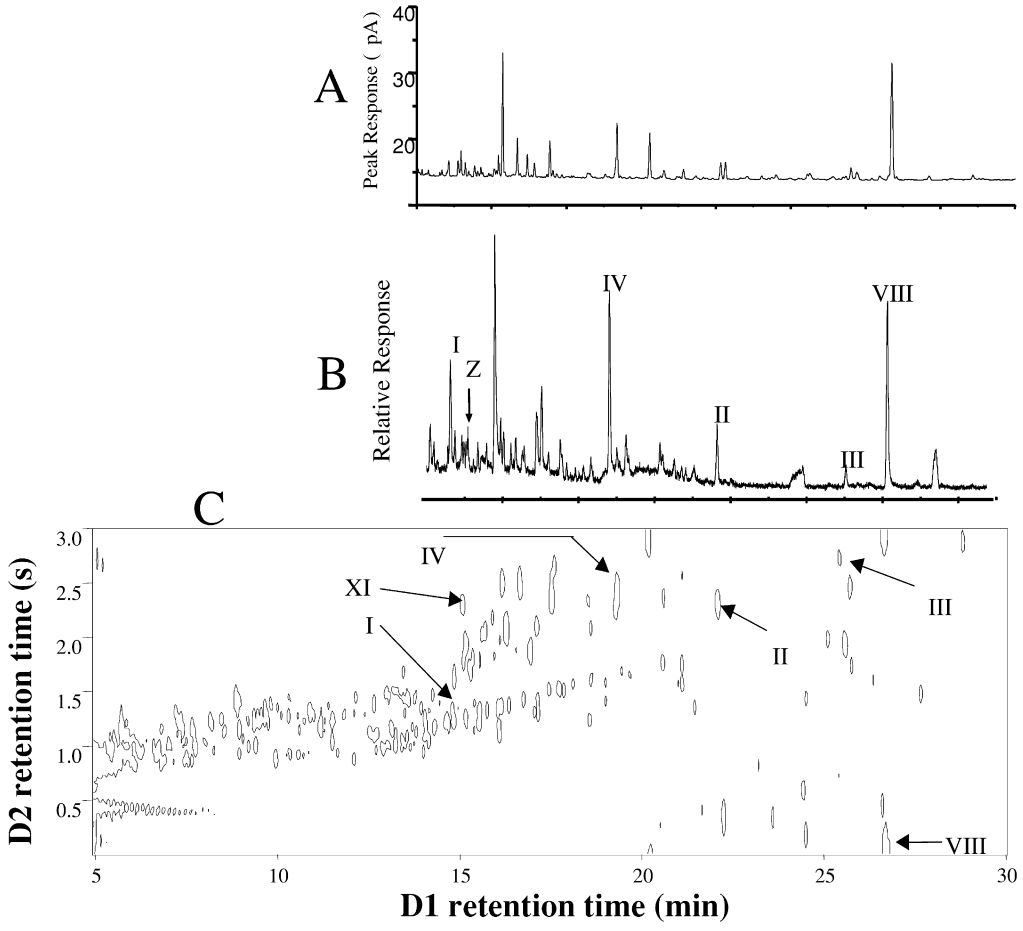

Fig. 7. Chromatograms of a greyhound urine extract taken 0–1 h after oral administration with prolintane. The partial GC and GC–MS runs are presented for the region where the metabolites elute. (A) GC–FID; (B) GC–MS; and (C) GC×GC–FID.

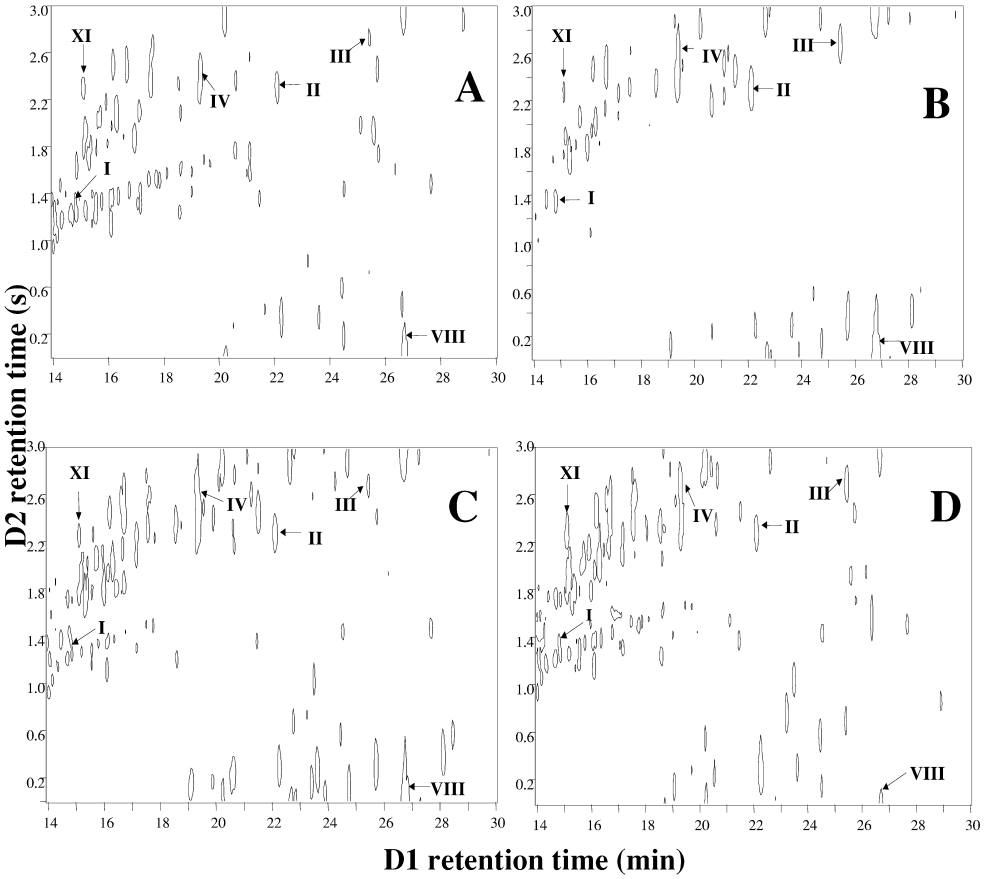

Fig. 8. Comparison of GC×GC contour plots for greyhound urine extracts taken after oral administration with prolintane. (A) 0–1-, (B) 2.3-, (C) 5.7- and (D) 24-h extracts. Contour plot levels are presented at 18, 20, 20 and 19 pA, respectively, in order to best illustrate the comparison of the samples.

presentation, but the clear positions of all target compounds makes their identifications straightforward in the case of GC×GC. Some indication of relative amounts is possible from these traces, by drawing various contour levels to estimate relative peak heights. Parent prolintane (peak I) is the smallest peak of interest, and suffers some overlap with another matrix component, however its position is known from analysis of the standard. Table 5 reports relative peak areas for the components in each of the samples, and as anticipated from drug metabolism and excretion processes, the low initial level of metabolites increases to a maximum then diminishes over time. Prolintane gives a small peak since it is not so readily excreted. In agreement with previously described excretion studies of prolintane [7,8], the parent drug is extensively metabolised and is only observed in urine at low levels for a short period after administration. Note that peak areas are reported for this study, rather than quantitative

Table 5
Peak areas (pA.s) for prolintane and metabolites measured in greyhound urine

Compound	Sampling time (h)			
	0–1	2.3	5.7	24
I	0.46	44.8	2.07	2.41
XI	2.93	169.73	69.84	15.91
IV	23.39	1197.5	485.8	15.12
II	7.14	399.5	34.45	16.66
III	7.70	47.97	3.98	10.69
VIII	60.95	1113.6	427.9	13.06

amounts, since authentic standards of the metabolites were not available, and peak areas serve to demonstrate that the method is capable of reliably producing excretion curve data under the GC conditions employed, for samples in which matrix interference is normally high.

4. Conclusions

This study has demonstrated a general approach to analysis of illicit drugs, which are part of routine screening interest to forensic toxicologists, by using GC×GC. Confirmation of absolute retention times on the D2 column was obtained by comparison with elution time determined by the targeted mode of operation of the cryotrap. A short (0.4 m) BPX50 column was used to minimise wrap-around. The capability of GC×GC to resolve the selected drugs from the background matrix of horse and greyhound urine is illustrated. Quantitative data may be readily obtained from both the targeted and GC×GC modes, with good calibration linearity and improved detection limits over conventional GC analysis. The reproducibility of the GC×GC result allows ready recognition of the drugs by their positions in the 2D retention plane, which permits screening analysis of a urine extract. This study suggests that GC×GC has potential in the area of routine drugs analysis, providing lower detection limits, good reproducibility and the possibility of sample fingerprinting.

Prolintane metabolites in dog urine were chosen as the worked example since this was previously used as a case study to demonstrate the central role of the information content of mass spectrometry in drug analysis [55]. The results for the earlier study (not shown here) are in general agreement with the present GC×GC results. For regulatory work, criteria for identification and legally defensible results may be based on the points of similarity and the information content of the data set, similar to a ‘‘fingerprint’’, with minimum standards for positive identification. It is necessary to match by some comparative technique (including computer algorithms) for points of similarity between a sample and a data set. For instance the matching of mass spectral data and retention time sets (GC–MS), or similarity matching of a component position within a specific two dimensional retention field (GC×GC) should permit more reliability in identification over single dimension GC analysis.

Acknowledgements

The authors wish to thank SGE International for providing support for capillary GC columns used in this research, and Agilent Technologies for GC facilities. The technical assistance of Paul Morrison is appreciated.

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