Rom J Leg Med 16 (2) 95 – 102 (2008)

Practical implications of GC and HPLC methods for the analysis of drugs of abuse in blood

*Catalin Dogaroiu**[1], Russell Sugden*[2]

Abstract: Rapid trace level quantitative determination of drugs and their metabolites is a challenge which is often driven by the need for same-day turn around of results for an increasing number of biological samples. This article reviews and compares GC and HPLC methods for the analysis of some important drugs of abuse, their metabolites and adulterants (cocaine, LSD, ketamine, benzodiazepines, strychnine) in blood, presenting the benefits and limitations of each method. It also outlines which of the two procedures is more suitable for general blood analysis performed when the drugs in the sample are unknown, as is often the case in the police work.

Key words: drugs of abuse, blood samples, GC, HPLC.

In the recent past urine was the sample of choice, due to the concentrating effects of the kidneys on metabolites, for the identification of drugs of abuse in both post and ante mortem samples. Improvements in detectors, chromatographic techniques and sample preparation have dramatically increased the number of substances which can be detected in human blood, as limits of detection have decreased it has become possible to detect substances present at ever smaller amounts which has in turn made blood an increasingly more viable alternative.

As a matrix, blood is relatively homogeneous and drugs can be detected in it after intake prior to metabolism [1]. Generally speaking, with the exception of glucuronides of Morphine and LSD analysis where LC methods are more suitable, the availability of deuterated analogues of drugs has facilitated the increased use of GCMS as a detection method and has made it the 'benchmark' technique in use.

HPLC Blood Analysis: comparisons and developments

Rapid trace level quantitative determination of drugs and their metabolites is a challenge which is often driven by the need for same-day turn around of results for an increasing number of biological samples [2].

**) Coressponding author:*
1) Catedra de Medicina Legala, Universitatea de Medicina si Farmacie "Carol Davila" Bucuresti,
2) University of Huddersfield, UK, Department of Chemical and Biological Sciences.

It is this need which, though perhaps not the most exciting research, a small improvements in the detection limits, precision and accuracy has the potential to have a vast impact on analyses performed on a daily basis, reducing the number of false negatives and positive is of obvious importance.

HPLC is as well known and understood as GC and is considered to be the 'counterpoint' to it as many compounds unsuitable for GC can be analysed using HPLC and vice versa. In HPLC the stationary phase is composed of extremely small porous particles which require a high pressure to move the mobile phase/analyte through the packed stationary phase in the column.

Important advantages to using small particle size including, high resolution, faster analyses and increased sample load capacity. [3] Typical columns are ~10 to 20cm in length and a fairly durable if treated properly, that is not with extremes of pH, contaminated biological samples or crude samples.

HPLC and HPTLC Though it is not possible here to perform and report directly on experiments to compare these two techniques, papers in print can be considered. This is typified by the work of Antonilli et al [4] have compared HPTLC and HPLC for the analysis of cocaine, cocaethylene and benzoylecgonine, in urine rather than blood however their results are indicative of the general trend. They found that HPTLC had LOD's and LOQ's for all three compounds to be 0.5 and 1.0µg/mL respectively [4] and that for HPLC the LOD for the three compounds to be 0.025µg/mL and the LOQ for benzoylecgonine and cocaine to be 0.2µg/mL and 0.1µg/mL for cocaethylene.

In addition to this HPLC was shown to have lower intra and inter day RSD values (intraday 0.79-5.05%, interday 1.19-10.64%) than those of HPTLC (intraday 1.03-12.60%, interday 1.56-16.6%), from which we can conclude, as expected, HPLC is both more precise and more accurate than HPTLC.

Comparative evaluation of HPTLC and HPLC found HPTLC to be ten times less sensitive the HPLC in quantitating benzoylecgonine and cocaine and five times less sensitive than cocaethylene. Worryingly, from the law enforcement point of view, it was found that three cocaine and one cocaethylene samples tested with HPTLC had given false negatives when re-tested with HPLC, this has been despite the addition of UV densitometric measurement to improve the HPTLC performance. Antonilli suggested, rightly, that HPTLC is acceptable only for high concentration samples and that it should not be discounted due to its affordability and speed as plate analysis can carry 10+ samples to be analysed in a few hours.

Essentially HPTLC is cheaper and faster though less accurate and precise than HPLC, from the academic/laboratory point of view HPLC wins hands down, however from the practical/immediacy point of view HPTLC is quite adequate where the levels of cocaine and its metabolites are expected to be quite high, immediately (a few hours) after the abuse of these substances.

This principle can hence be extended to a much wider range of analytes; TLC methods are 'good enough' as apposed to 'outstanding'. HPTLC and TLC are ideal for samples gathered from a 'midnight raid' whereas HPLC is perhaps better suited for randomised testing. Though it must be conceded that the nature of blood would make this method unsuitable without some form of prior preparation.

Developments with small samples and DAD. Though it may not be considered especially critical, the refinement of currently available methods is of great importance to Chromatographic science in general and in particular the applications of such to forensic

science. At the beginning of 2004, French researchers Duverneuil C. et al [5] published research in which they describe the use of photodiode array detection (DAD) HPLC for the determination of strychnine in blood.

Though primarily a rodenticide and currently out of favour as such, it has be found as an adulterant to street drugs, strychnine in Heroin and Opium has been used to identify its source [6] which can be highly important from the point of view of law enforcement. Lethal doses for strychnine begin at 2mg/L (2µg/mL) upwards depending on the victim's constitution.

Duverneuil found that TLC, GCMS, and HPLC methods had all been widely described in great detail, all of which required at least 1mL of sample [5] something that is not always available. Using liquid-liquid extraction and HPLC/DAD they developed a new method requiring only 0.1mL of sample for both identification and quantification. The limit of detection for their new method was found to be 0.06mg/L (0.06µg/mL).

Duverneuil et al detected strychnine quantitatively at 25mg/L (25µg/mL) in a blood sample from a French self- poisoning suicide. The LOD of the signal to noise ratio was 5:1 (rather than 3:1). "The use of DAD detection increases the specificity of the method allowing the identification of the unknown compounds by comparing their UV spectrum with those of a library" [5] The reported improved method, thought to be by the contributing authors to be the first reported method requiring only 0.1mL of sample [5], will no doubt stimulate future research, hopefully lowering the amount of sample required to obtain accurate, repeatable, useful results on the analysis of body fluid samples for the presence of not just strychnine but a wide range of drugs of abuse.

LSD analysis with HPLC. Lysergic Diethylamide presents a series of interesting challenges to the analytic chemist, the active oral dose is usually around 50µg (though 500µg has been reported [7]), the active blood concentration is very low 2µg/kg (2ng/g) as a result of this LSD analysis has routinely been performed on urine as its metabolites can be present at substantially higher concentrations than in the blood. As a result most publications describe methods for urinalysis.

Though it is possible to attempt GC analysis of LSD this is unadvisable as it has low volatility and thermal stability at GC temperatures and if it is not derivatised by silylation it will become irreversibly bound to the GC column. In short GC methods of analysis are unsuitable for LSD detection and TLC methods (though cheap and fast) lack the specificity and control required. Though it might be said that with 'complete unknown' samples, running a TLC plate and identifying LSD is substantially cheaper than running/ruining a GC to uncover the same fact.

Hence HPLC is the favoured technique of analysis, though it is with LC/MS/MS where we find sufficient selectivity and sensitivity to perform it. Only a limited number of papers have outlined methods for analysis of blood. Both Faverotte [8] and Canezine [9] have out forward one-step liquid-liquid extraction from biological samples (i.e. urine and blood) prior to LC/MS/MS which describe sensitive methods with acceptable sample size. However these two methods require extraction with chloroform and diethylether respectively (a known carcinogen and highly flammable liquid) and Johansen et al [10] have described a new one-step liquid-liquid of 1.0g of whole blood (or 1.0mL urine) using 1.0mL of butyl acetate.

The method put forward by them claims to have an impressively low LOQ of 0.01µg/kg (0.01ng/g) of LSD in both blood and urine. In addition the method was applied to a real case, where blood concentrations were found to be 0.21 and 0.44µg/kg of LSD and iso-LSD respectively [10]. They also outline the importance of good chromatographic separation

of isomers of LSD as these can produce similar (almost identical) fragmentation patterns in MS. Due to the unsuitability of GC methods in LSD analysis developments in HPLC methods can be of great significance.

Ketamine analysis with HPLC. Aboul-Enein and Hefnawy [11] have developed a reduced analysis-time determination for Ketamine (2-(2-chlorophenyl)-2 methyl aminocyclohexanone) and two of its metabolites using monolithic columns without the reduction of chromatographic resolution, from human plasma.

As outlined in the above paragraph, the use of a monolithic silica column allows higher flow rates without significant increases in backpressure. As above the main advantage of monolithic columns is that analysis can be completed quickly without adversely effecting resolution. Aboul-Enein and Hefnawy employed SPE extraction. The process outlined in their paper showed good linearity from 25-2000ng/mL, had an LOD and LOQ of 7.5ng/mL and 25ng/mL respectively [11] as in the analysis of BZD's above, their method reduced the amount of mobile phase used overall (though the amount of mobile phase per unit time is increased) and reduced the run time to around 4min [11] without affecting the separation between the peaks. They have also suggested that "…most chromatographic assays on standard reversed phase columns can be rather easily transferred to commercially available monolithic LC phases" [11]. The increased number of papers being published making use of monolithic suggests that this is an area of interest, likely to yield interesting results in the near future.

BZD analysis with HPLC. BZD (Benzodiazepines) are a group of compounds known for their sleep inducing and anxiolytic effects which are also known to be addictive. As with LSD, BZD's require a derivatisation step (to increase their volatility) in order to proceed with GC analysis (see below), though several chromatographic methods are reported in current literature using ECD, NPD and most frequently MS detectors.

The necessity of the derivatisation step has ensured that HPLC is also used in detection of BZD's, however these methods can be time consuming due to the flow rates involved. Mahjoub and Staub [12] outlined a method for the analysis of BZD's using HPLC/DAD with Chromlith™ columns. More recently Bugey and Staub [13] have developed this method further applying liquid-liquid extraction of BZD's from whole blood samples (1mL) using n-butyl chloride followed by reversed phase HPLC with Chromolith™ silica rod column, the bipourous structure of the rod creating a large surface area allowing greater permeability [14] which allows the use of high flow rates without loss of performance [14, 15] essentially allowing the detection of these compounds much faster without loss of sensitivity.

Amongst the range of BZD's tested with this method, Diazepam (the most widely commercial available) was detected at LOQ of 30ng/mL (all the BZD's investigated were found to have LOQ's between 20-30ng/mL), compounds were tested over a range from 30 to 5000µg/L and found to have a linear relationship (correlation coefficient greater than 0.99), the interday RSD was found to be around 5% and most impressively the duration was less than 4min, "reducing analysis time by factor of 5 compared with assays based on packed HPLC Columns…" [13]. Surprisingly no LOD is given for this method by the authors. Overall this method can be used for the analysis of 20 BZDs at LOQ within the therapeutic range of use [13], they additionally found that this method could be applied to compounds such as methadone also.

Most importantly of all though, the reduction in the time required to produce reliable result to less than 4min is significant in practical terms of the number of analyses that can be performed with 'the working day' by, theoretically a factor of five, a substantial amount. As

before we see that seemingly insignificant advances in the application of current techniques can have the potential to dramatically alter the results gathered from 'the field'.

GC Blood Analysis: Comparisons and developments

Gas chromatography methods, especially GCMS, have come to prominence in routine lab work due to its versatility, ease and cost of maintenance and operation, and separation efficiency. Generally, provided the compound of interest falls with the parameters of GC analysis in terms of volatility etc, analysis can easily be performed, however it is often the case that the compound of interest has to derivatised prior to analysis to prevent it from becoming irreversibly bound to the column and/or to make it volatile at GC temperatures.

The usually approach taken by the analyst is to consider the chemistry of the compound of interest and then proceeded with the 'best' technique as appropriate, be that GC or HPLC. It should be noted that, the purely chemical considerations alone are not always on the analysts mind, familiarity with the method, run time required, accuracy required, reproducibility of result and, unfortunately cost can sometimes be a factor hence the number of papers which deal with questions of reducing volumes of solvents required and so forth.

BZD analysis with GCMS. BZD's (Benzodiazepines) can be analysed by both GC and HPLC, due to their chemistry BZD's require derivatisation in order to facilitate analysis. T.Gunnar et al [14] have reported on the efficiency of the most commonly available silylating reagents for derivitising BZD's prior to GCMS analysis and found that by derivitising BZD's increased thermal stability, sensitivity and produced specific mass fragments in the mass spectra. They evaluated the silylating agents for determine the optimal derivative for GCMS of BZD's extracted from whole blood. Since BZD's have both acidic hydroxyl and/or basic amine groups in its molecular structure which present at extreme pH values in ionic forms [14]

T. Gunnar et al found the LLE and *n*-butyl acetate allows rapid and efficient extraction when compared to SPE methods, they only major fault of using LLE is the relatively high boiling point of the solvent they used (126°C) however, as they point out this is not necessarily a problem as BZD's (and hypnotics in general) "…evaporate at significantly higher temperatures" [14]. In addition they note the practical difficulty in maintaining the anhydrous conditions needed for silylation reactions, they even go so far as to point out the effects of increased laboratory humidity at certain times of the year and that, if necessary silylation should be performed under a nitrogen atmosphere. Also Silylating reagents are carcinogenic and expensive. Using the method outlined in their paper they found the LOD and LOQ of Diazepam (the most commonly used and abused BZD) to be 2ng/mL and 100ng/mL. Of the BZD's tested with this method LOQ's ranged from 20-100ng/mL with half of those tested having an LOQ of 100ng/mL.

Comparison of SPE procedures for GC. The comparison outlined by de la Torre et al [15] between Chem Elut and Bond Elut Certify SPE columns for their recovery, precision, sensitivity and efficiency in drug separations from blood, (as part of STA, systematic toxicological analysis) followed by GC-NPD is not only applicable to gas chromatography methods but also other techniques, such as HPLC-DAD and GC-MS [15], as SPE is a preparative, rather than principle chromatographic, procedure. Their paper addresses issues facing the 'field analyst' who is looking for the most efficient, reliable methodology to produce reproducible results.

In STA, one of the most frequent general analysis methods used by forensic and non-forensic investigators, as the substance may not be known at the start of the analysis a general

extraction procedure is required. In the past LLE dominated but with the development of SPE it has grow in frequency of use, principally due to the fact the chance of an emulsion forming is greatly reduced, the volumes of solvent required are lower and, most importantly, it is possible to automate the process [15] allow the analyst to perform other takes while this is happening. As outlined before, de la Torre et al note the emphasis of research on 'clean' biological samples such as plasma, serum and urine rather than whole blood. It is whole blood which in practice is the most frequently encountered in forensic cases and is increasingly seen as sample matrix for 'drug abuse' case. Several papers are cited by them in support of this [15]. The Chem elut column process is very similar to conventional LLE, a diatomaceous earth acts as a support for the aqueous phase, the high surface area ensures that organic eluents remain immiscible with the aqueous matrix [15] (eliminating emulsion problems), however where STA is concerned it is probable that more than one separation will be required due to the fact the acidic, basic and neutral molecules will doubtless be present, requiring large volume of solvents.

The second type is the Bond Elut Certify column which is a "…mixed-mode bonded silica SPE extraction support containing hydrophobic chains and strong cation-exchange moieties…" [15] giving it the ability "…to retain acidic, neutral, basic and amphoteric drugs on one column…"[15] The advantages of this for whole blood analysis are obvious. De la Torre et al point out the unusually high number of nitrogen containing compounds that are drugs facilitates the use of the specific NPD detector type, and as blood does not usually contain drugs (or their metabolites) at the same concentrations as urine. In total the compared the two extraction procedures and found that bond elut certify columns "resulted in more advantages besides the detection of drug, such as better and more easier reproducible recoveries, cleaner extracts, better sensitivity, and less solvent consumption and disposal" [15] This assertion is supported by the data they present in the paper. They conclude that the use of the bond elut column resulted in more reproducible recoveries, cleaner extracts and reduced solvent consumption and that it is superb alternative.

This study, prompted by the use of both procedures in the lab of the authors, is also applicable to other chromatographic methods not just gas chromatography. It is far from being irrelevant as it outlines the pros and cons of each of these two methods, it also goes on the outline which of the two procedures more suitable for general blood analysis performed when the drugs in the sample are unknown, as is often the case.

Comparison between GCMS, 2-Dimensional GCqMS and 2-Dimensional GCTOFMS.

In a particularly technical and in depth paper S.M.Song et al [16] have compared these three techniques and have concentrated on the technical aspects of each. They have outlined that the initial and maintenance costs involved in TOFMS GC methods as well as the huge amounts of data (and processing time associated with it) generated require relatively modern computers [16] they cite one paper which records a 7 hour processing time. Hence they conclude that while accurate 2D GC-TOFMA is currently unsuitable for routine laboratory work, the data acquisition rate of up to 500Hz over 0-1000 mass range [16].

They found that the use of multidimensional (that is to say more than one column of different permeability/polarity in sequence) GC is advantageous in distinguishing between various large molecular mass drugs. They main conclusion of this work was to show that given the scarcity of TOFMS currently in use, the addition of quadrupole mass spectrometry to 2DGC would be an attainable improvement to those currently using 2DGC regularly and that "…developing a qMS approach that yields GCxGC-qMS interpretation for both

identification and quantitative purpose should be an attractive option to many current users" [16].

Conclusions and points of interest

Having looked at both GC and HPLC method developments for BZD detection from samples of whole blood, it is possible to compare these two methods. Based on just the two studies listed here, it is possible to say that for BZD's HPLC is by far and away the more superior of the two. The need to silylate BZD's (which is often required for GC analysis) with expensive, carcinogenic, moisture sensitive reagents [14] when compared with the method described by Bugey and Staub [13] which is much easier to perform, makes HPLC more attractive.

However the fact the HPLC method of Bugey and Staub, is not only very fast (in comparison to older HPLC techniques) but has reported LOQ's routinely significantly lower than that of T.Gunnar's GCMS method [13, 14]. The comparative ease of this HPLC method in comparison to the GCMS strongly indicates that HPLC would be the first choice for analysis, if this trend is maintained across a wide range of analytes. The developments in HPLC are enabling it to compete with GC in terms which it was previously unable to do.

The decreased sample size required for the detection of strychnine in blood (as put forward by Duverneuil et al [5]) may not seem at first glance to directly have an impact on the detection of drugs of abuse, however as strychnine is known to have been used as an adulterant in Heroin manufacture it may be possible to trace several Heroin 'samples' to a single source which can be greatly useful to law enforcement agencies. As 0.1mL is a very small amount of initial sample, were it to become possible to extract a sample from drops of blood of this side, the method developed by Duverneuil could prove to be a no small importance.

Though it may not seem to be relevant, the comparison between two SPE procedures (bond elute certify and chem elute columns) available by de la Torre [15] showing that the development of bond elute certify mixed phase columns as a 'one column' method proves to be of significant importance. As it is directly applicable to the daily laboratory analysis. Though it should be noted that in the method they outline GCNPD is used and that some drugs cannot be detected by NPD despite the fact the there would be minimal interference from non-nitrogen containing compounds. Their work suggests that by and large the bond elute column would be the SPE method of choice, though further research would be necessary to determine the certainty of this.

The development of monolithic columns [13, 14, 16] which can be run at increased flow-rates without adversely affecting peak separation and resolution, it is obvious therefore that this development, which if the promise of research can be translated into practical applications will doubtless have a dramatic impact on 'law enforcement analysis' where there is always a pressure to turn round, accurately, as rapidly as possible samples as small as possible. Were it possible to perform ten analyses in the same time that it previously took to perform five, in a situation were resources and manpower are limited the benefits can be substantial.

The use of GCxGC-TOFMS outlined by Song et al [16] indicates the direction Gas Chromatography detection research in the near future but as they have highlighted drawbacks associated with it such as the prohibitive cost of TOFMS, computing power required for data

analysis and the need for developments in "Molecular Libraries" indicate that it may yet be some time before this method is widely and deeply distributed.

Overall, the general trend is that developments in both GC and HPLC techniques is as would be expected, continually ongoing and that there is a great deal of research being undertaken into 'small developments' into aspects of the analytical process, which may at first glance appear to be of little importance, will have a dramatic impact on routine laboratory work. Small advances in the analyst's choice of technique, sample preparation and recovery, even reducing the volume of solvent required, should all contribute to increasingly accurate, precise and reliable results at, hopefully increased turn around and lower cost. This ability to operate reliably at increased speeds will in turn be of great benefit to law enforcement in general and the war against drugs in particular.

References

1. Moeller MR, Steinmeyer S, Kraemer T, Determination of drugs of abuse in blood. J Chromatogr B 1998 713: 91–109.
2. Barbarin N, Mawhinney DB, Black R, Henion J. A fast LC-APCI/MS method for analyzing benzodiazepines in whole blood using monolithic support. J Chromatogr 2003 B 783: 73-83.
3. Kazakevich YV, LoBrutto R. HPLC for Pharmaceutical Scientists. 1st ed. Oxford (UK): Wiley-Interscience; 2007.
4. Antonilli L, Suriano C, Grassi MC, Nencini P. Analysis of cocaethylene, benzoylecgonine and cocaine in human urine by high performance thin layer chromatography with ultraviolet detection. A comparison with high performance liquid chromatography. J Chromatogr 2001 B 751: 19-27.
5. Duverneuil C, de la Grandmaison GL, de Mazancourt P, Alvarez J-C. Liquid chromatographic/photodiode array detection for determination of strychnine in blood: a fatal case report. Forensic Sci Int 2004 Apr 20;141(1):17-21.
6. Wijesekera AR, Henry KD, Ranasinghe P. The detection and estimation of (A) arsenic in opium, and (B) strychnine in opium and heroin, as a means of identification of their respective sources. Forensic Sci Int 1988; 36(3-4):193-209.
7. Schneider S, Kuffer P, Wennig R. Determination of lysergide (LSD) and phencyclidine in biosamples. J Chromatogr B Biomed Sci Appl 1998 Aug; 713:189-200.
8. Faverotte D, Frison G, Maietti L, Tedeschi L, Ferrara SD. Ann. Toxicol. Anal. 2002;14(3): 201
9. Canezin J, Cailleux A, Turcant A, Le Bouil A, Harry P, Allain P. Determination of LSD and its metabolites in human biological fluids by high-performance liquid chromatography with electrospray tandem mass spectrometry. J Chromatogr B Biomed Sci Appl 2001 Dec 5;765(1):15-27.
10. Johansen SS, Jesen JL. Liquid chromatography-tandem mass spectrometry determination of loperamide and its main metabolite desmethylloperamide in biological specimens and application to forensic cases. J Chromatogr B Analyt Technol Biomed Life Sci 2004 Nov 5;811(1):31-36.
11. Aboul-Enein HY, Hefnawy MM. Liquid chromatographic high-throughput analysis of ketamine and its metabolites in human plasma using a monolithic silica column and solid phase extraction. Talanta 2005;65(1):67-73.
12. Mahjoub A, Staub C. Simultaneous determination of benzodiazepines in whole blood or serum by HPLC/DAD with a semi-micro column. J Pharm Biomed Anal 2000 Aug 15;23(2-3):447-458.
13. Bugey A, Staub C. Rapid analysis of benzodiazepines in whole blood by high-performance liquid chromatography: use of a monolithic column. J Pharm Biomed Anal 2004 May 28;35(3):555-562.
14. Gunnar T, Ariniemi K, Lillsunde P. Determination of 14 benzodiazepines and hydroxy metabolites, zaleplon and zolpidem as tert-butyldimethylsilyl derivatives compared with other common silyating reagents in whole blood by gas chromatography-mass spectrometry. J Chromatogr 2005;818:175–189.
15. de la Torre CS, Martinez MA, Almarza E. Determination of several psychiatric drugs in whole blood using capillary gas–liquid chromatography with nitrogen phosphorus detection: comparison of two solid phase extraction procedures. For Sc Int 2005 Dec 20;155(2):193-204.
16. Song SM, Marriott P, Wynne P. Comprehensive Two-dimensional Gas Chromatography – Quadrupole Mass Spectrometric Analysis of Drugs. J Chromatogr A 2004;1058:223-232.