The Role of Chromatography in Bioanalytical Studies

by The Disinterested Analyst

The accurate determination of the concentration of a drug candidate and it's resulting metabolites in biological fluids plays an essential in the drug discovery, preclinical and clinical development programs. The drug concentration values determined are used to calculate the pharmacokinetics properties of the candidate drug molecules; such as Area Under the Curve (AUC), T 1/2, Cmax and elimination constant. In discovery these values are used to select the most promising candidate compound, from a library or parallel synthesis experiment, to be carried forward into development. In development the pharmacokinetic values derived from both DMPK and safety assessment studies are used to provide evidence of the dosed compound exposure, (in safety assessment studies) evaluate the effect of increased drug dosing on compound exposure and estimate the appropriate dose to be used in the initial human clinical trails. At this point the metabolism of the compound in mammalian species, such as rat, dog and mouse is also extensively studied and accurately defined. Again this requires the accurate measurement of drug and metabolite concentrations; normally performed using a radio labelled isotope of the drug. During the initial phase I clinical studies the derived drug concentration values are used to determine the pharmacokinetics and exposure of the drug in health human volunteers. As these values are used to measure the effect of increased exposure to the drug and give guidance to the clinical team with respect to the next dose, rapid assay turnaround is required to deliver results in a 24 hour time period or less. As the early dosing in man is at extremely low levels the assay must have sufficient sensitivity to accurately determine the elimination phase of the pharmacokinetics curve if correct values for half-life, AUC and elimination constant are to be determined.

Liquid chromatography coupled to UV [1] or sometimes fluorescence or electrochemical detection [2-3] has been successfully employed to support drug discovery, development and clinical trial studies. However the need to complete analyte resolution from the endogenous components in biological matrices resulted in run times in the order of 20-40 minutes which in turn limited the ability to provide fast turn round of results. Whilst the use of fluorescence and electrochemical detection provided assays in the low ng/mL and pg/mL level when the analyte chemistry allowed, the majority of the time assay sensitivity was limited to the mid ng/mL level. The use of solid phase extraction allowed an analyte pre-concentration with a clean up step, simultaneously improving assay robustness and increasing sensitivity to the low ng/mL range [4]. In general, with highly potent compounds dosed at very low levels, assay sensitivity could become a limiting factor for project progression. To develop a method with the required sensitivity for these low dosed compounds or compounds with low circulating levels such as inhaled products could take a significant amount of laboratory time with derivitization schemes being required in order to impart the necessary chemical properties upon the analyte molecule to allow for detection by a more sensitive technique; fluorescence being the most popular [5-6]. An alternative approach sometime adopted is the use of radio immuno assay (RIA) this approach, which involves the growing of antibodies to the drug molecule (normally using a host such as the sheep) can be very sensitive. Although sensitive RIA does have several limitations which have limited its utility namely, lack of specificity via cross talk, poor linear dynamic range and most importantly the time taken to raise the antibodies and develop the methodology [7]. The development of the atmospheric pressure ionisation (API) source in the late 1980's allowed the direct interfacing of liquid chromatography with mass spectrometry [8]. The use of mass spectrometry as a detector imparted several critical benefits on liquid chromatography [9]. The orthogonal nature of mass detection allowed for an added degree of specificity into the analytical process; the use of selected ion monitoring (SIM) or multiple reaction monitoring (MRM) reduced the need for complete resolution of analytes in the chromatography method allowing for shorter run times. The versatility of the detection process removed the need for compound derivitization, or the need for antibody generation making method development faster. Finally the overall detection process was more sensitive than UV (and in most cases fluorescence and electrochemical detection) allowing for lower limits of sensitivity [10]. As previously mentioned, the specificity of the MS detection process allowed shorter analytical run times, higher assay throughput and chromatographic methods requiring less resolution [11]. Indeed some scientists even removed the chromatography process completely performing on-line SPE with small cartridges packed with chromatographic material. Raw plasma was diluted and injected on to the extraction cartridge where it was washed with buffer and organo-aqueous mixtures to remove endogenous material before elution using an organic solvent. The resulting eluent from the SPE process was directed into the mass spectrometer [12] without employing any further LC separation. This methodology allows for simple rapid analysis of pharmaceutical compounds in plasma or serum with total analysis times of just 5-8

minutes from sample extraction to result with sensitivities in the low ng/mL range.
This approach was further simplified by replacing the small cartridges used in the on-line SPE process with short analytical columns (1 x 50 mm) packed with large diameter chromatographic material (30-50 µm). Like the on-line approach the serum/plasma samples were diluted with aqueous buffer and injected onto the chromatographic column which was then eluted with a rapid 1 minute gradient and flow rates as high as 5 mL/min (see Figure 1).

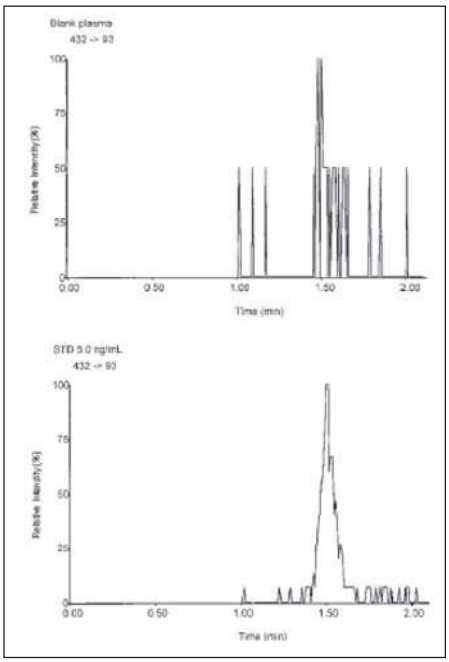

Figure 1. Sample chromatograms of blank plasma (top) and of plasma spiked with standard at 5 ng/ml (bottom). Chromatographic conditions: Column Explorer Turbo-C18 (50 x 1 mm, 50 m); mobile phase a 0–95% acetonitrile gradient from 0 to 0.9 mins; temperature 40 °C, injection volume 50 µl. Reproduced from reference 13

This approach, sometimes called Turbulent Flow LC, allows for assays with analysis times in the region of 1 minute [13-14]. Further multiplexing of the columns with a MUX interface to the MS allowed for the processing of 8 samples every two minutes [15]. Attractive though this approach initially appears it suffered from some serious drawbacks. The high volumetric flow rates employed required that most of the column eluent be directed to waste, reducing assay sensitivity. Most assays reported using this approach were in the 5-10 ng/mL range. The most severe drawback of this approach however is the lack of chromatographic resolution. As reported by Jemal et.al. [16] this lack of resolution can produce inaccurate results if the target analyte is not resolved from drug metabolites present in the sample. Metabolites such as N-oxides and glucuronides are thermally labile and can

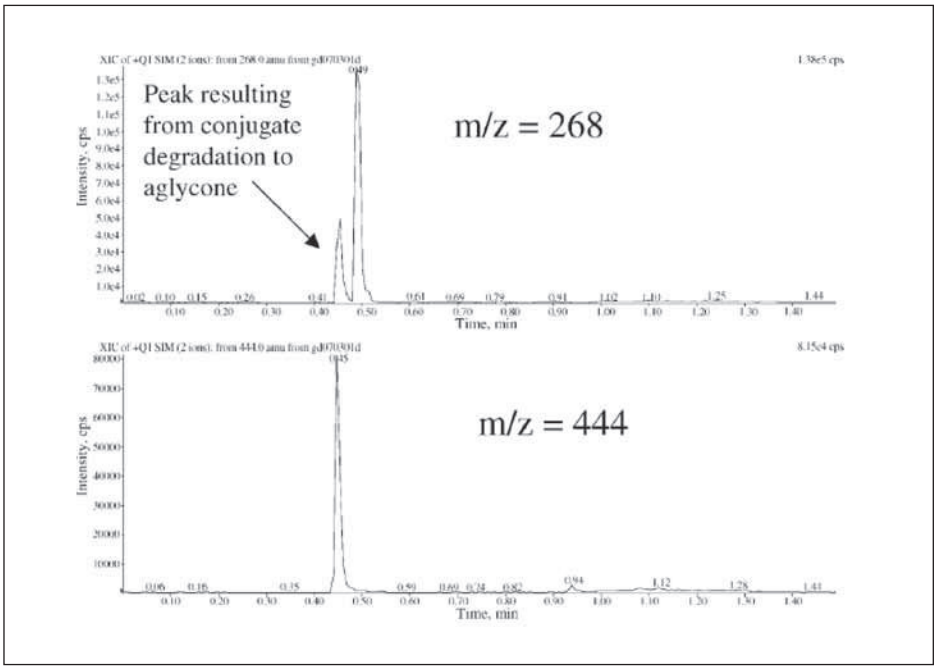

Figure 2. Extracted ion chromatograms for the analysis of AZT and AZT-glucuronide. Reproduced from reference 17.

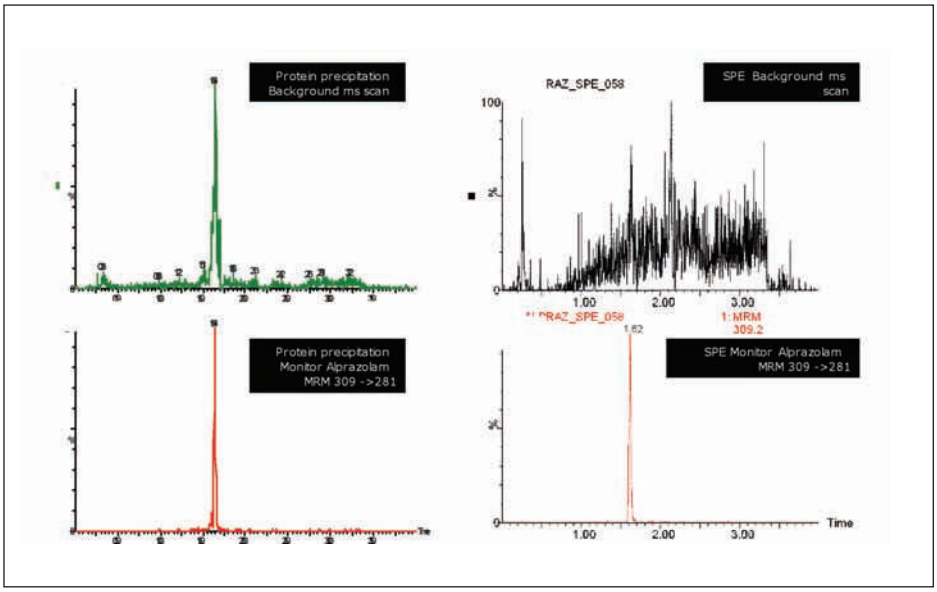

Figure 3. The use of solid phase extraction to remove co-eluting ions in a bioanalytical assay. Reproduced from reference 21.

be converted to the aglycone in the MS source. Without chromatographic resolution this will means the metabolite signal is indistinguishable from the analyte in the mass spectrometer, even with the specificity of MS/MS detection. This effect was also observed by Ayrton et.al. for the analysis of AZT and AZT glucuronide in plasma [17] (see Figure 2). Here they clearly demonstrated how this lack of resolution compromised the assay's performance. The authors solved this problem by replacing the high particle size column with a silica monolith, although the flow rate was still in the region of 4 mL/min and required flow splitting into the MS source. The extra resolution of the monolith material completely resolved the two analytes and allowed the direct injection of plasma onto the column. These on-line approaches remain a viable option for bioanalysis. Throughput is excellent and for assays where low levels of detection are not required they provide an attractive approach. The small column diameter available for the monolith technology combined with their 4-5 µm particle like LC performance make these, in the opinion of the author the best option for direct plasma analysis.
The drive for improved productivity and faster analysis time had forced the bioanalytical scientist to trade off sensitivity and specificity with throughput. The drive for more sensitive assay had mainly focussed upon the performance of the mass spectrometer and the impact of the chromatography process on

sensitivity was not fully exploited. However the benefits of the chromatography process on assay sensitivity are well known. In the late 1990's Jorgenson showed how sub 2 µm particle LC operated at elevated pressures could be used to provide higher resolution separations with greater sensitivity [18]. The latest generation LC technology has allowed the chromatographic performance of sub 2 µm particle LC to be fully exploited. Whilst these sub 2 µm particle materials have been commercially available since the early 2000's [19], their true chromatographic potential was not truly realised until dedicated LC systems became available offering higher pressure capabilities, reduced delay volumes and optimised dispersion characteristics [20]. The LC/MS community was quick to take advantage of the extra resolution generated by these sub 2 µm LC systems, using the narrow chromatographic peaks generated to improve MS sensitivity and reduce analysis times [21] (e.g. Figure 3). Indeed, of over 400 papers published on this sub 2 µm chromatography the vast majority relate to MS and MS/MS analysis. The higher throughput assays allowed by these materials was illustrated by Hayes et. al. [22]. In this paper they showed how analysis times can be reduced by a factor of 2-3 whilst improving assay sensitivity by a factor of at least 2. The assays robustness was demonstrated to be at least as good as that obtained with conventional HPLC/MS/MS. Houghton and Grace reported that with good laboratory practices over 3000 injections of protein precipitated plasma can be obtained in each LC column [23]. The benefits of the resolution obtained from these smaller particle columns was also illustrated by Houghton and Grace. They showed how the extra separation power was used to resolve the analyte of interest from unexpected co-eluting endogenous compound (and also resolution of related geometric isomers) in an epidemiological study to increase the number of analytes quantified. Over the last 3 years there have been several FDA discussion documents and guidelines relating to bioanalysis and bioanalytical assays. These documents have focussed on three major areas i) matrix effects, ii) incurred sample reanalysis and iii) metabolites in safety testing (MIST). All of these factors have an influence on bioanalytical challenges faced by today's scientists working in this field. It is well known that the sample matrix can significantly influence the sensitivity and robustness of a method [24]. The co-elution of endogenous components in the matrix, particularly the phospholipids present in plasma can result in ion suppression which in turn can reduce assay sensitivity and precision. Solid phase extraction has been exploited to remove these interfering

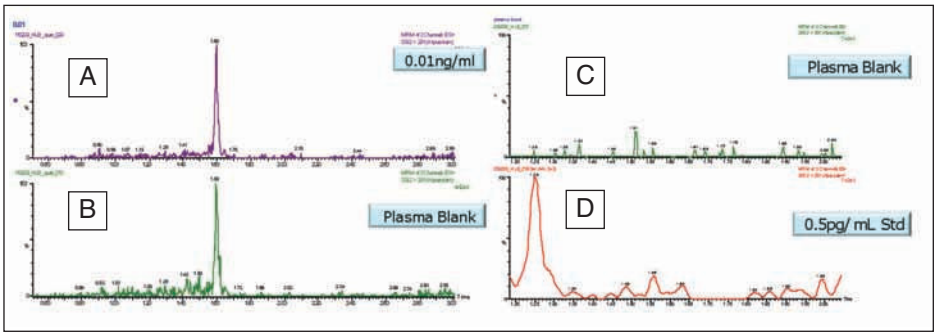

Figure 4. The resolution of an interfering matrix peak from the alprazolam analyte by changing chromatographic conditions. The initial 10 pg/mL and plasma blank are shown in chromatograms A and B. The improved separation with new chromatography conditions are shown in C and D, with the blank and 0.5 pg/mL blank

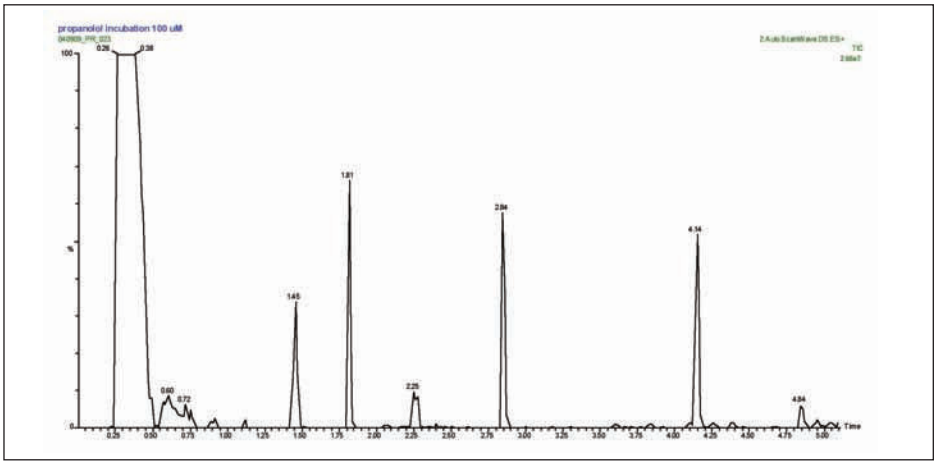

Figure 5. Sub 2 µm LC/MS/MS of propranolol metabolites from in vitro incubation. The peak at 4.1 minutes is the propranolol peak, the peak at 2.8 minutes is the hydroxy metabolites and the peaks at 2.2, 1.8 and 1.5 are the hydroxy-glucuronide metabolites. Reproduced from reference 25.

compounds and improve assay sensitivity (Figure 3). In this example the assay sensitivity was limited to 10 pg/mL using simple protein precipitation due to an interfering matrix peak. By employing an SPE process the assay sensitivity was improved by removing the interfering matrix peak. In this case the matrix effect and analyte response were simultaneously monitored using MRM and full scan MS detection. However solid phase extraction alone can not necessarily always address the matrix issues encountered in developing a high sensitivity example. As illustrated in Figure 4 changing the LC conditions can often be sufficient to address this issue. In this case varying the organic modifier allowed the assay sensitivity to be lowered by resolving the analyte of interest from a interfering matrix peak.
The recent regulatory guidelines relating to drug metabolites in safety testing has placed greater emphasis on resolving and detecting metabolites during bioanalytical studies. Recent publication by Leclercq et.al. discussed the importance of metabolite resolution from the parent compound and from each other [25]. In this paper they pointed out that incomplete resolution of isobaric metabolites could cause needless extra work to be carried out. As pointed out by Leclercq, the extra resolving power of sub 2 µm particle LC can address this issue without having to resort to longer analysis times or more complex method development. The data displayed in Figure 5 shows how the high resolution of these small particles allows for the complete resolution of the metabolites of the beta-blocker propranolol in just 5 minutes. Here we can see that the three hydroxy-glucuronides are clearly resolved, allowing for accurate determination of their concentrations. As the science of bioanalysis continues to the evolve and react to new challenges from regulatory guidelines, new compound classes and smaller sample sizes (e.g. from tail bled rodent studies), the role and application of chromatography will continue to evolve. As we look to the future we can expect to see that the use of chromatography will play a major role in delivering high sensitivity highly specific and fast analysis. The role of microscale separation may become more important as the sample size is reduced. The use of micro separations devices on a cartridge or integrated device may allow bioanalysis to be moved into the clinic. This will allow fast turnaround of results and remove the need for costly sample transfer. This will become especially important in epidemiological

studies where the samples run into the tens of thousand per year. As the importance of detecting metabolites, impurities and co-administered compounds increases the need for higher resolution will increase the drive for more efficient separation processes (longer columns or smaller particles). Supercritical fluid LC holds the potential for high resolution, fast sample analysis and could well be the key to solving these issues as well as dramatically reducing solvent usage and disposal costs.

References

1. *Liquid chromatographic high-throughput analysis of ketamine and its metabolites in human plasma using a monolithic silica column and solid phase extraction. Aboul-Enein HY, Hefnawy MM. Talanta. 2005;65(1):67.*
2. *Column-switching high-performance liquid chromatographic determination of clarithromycin in human plasma with electrochemical detection. Choi SJ, Kim SB, Lee HY, Na DH, Yoon YS, Lee SS, Kim JH, Lee KC, Lee HS. Talanta. 2001;54(2):377.*
3. *The benefit of the retrofitting of a conventional LC system to micro LC: a practical evaluation in the field of bioanalysis with fluorimetric detection. Roy S, Rieutord A, Zhou JY, Baillet A, Prognon P, Chaminade P. Biomed Chromatogr. 2003;17(5):297.*
4. *High-performance liquid chromatographic assay for 2'-deoxy-3'-thiacytidine in human serum. Harker AJ, Evans GL, Hawley AE, Morris DM. J. Chromatogr. B Biomed. Appl. 1994 657(1):227.*
5. *A sensitive post-column photochemical derivatization/fluorimetric detection system for HPLC determination of bisphosphonates. Pérez-Ruiz T, Martínez-Lozano C, García-Martínez MD. J. Chromatogr A. 2009;1216(9):1312.*
6. *General strategies and selection of derivatization reactions for liquid chromatography and capillary electrophoresis. Krull IS, Deyl Z, Lingeman H. J. Chromatogr. B Biomed. Appl. 1994;659(1-2):1.*
7. *A sensitive radioimmunoassay, incorporating solid-phase extraction, for fluticasone 17-propionate in plasma. Bain BM, Harrison G, Jenkins KD, Pateman AJ, Shenoy EV. J. Pharm. Biomed. Anal. 1993 (7):557.*
8. *The Origins of Ion Spray Liquid Chromatography-Tandem Mass Spectrometry. Henion JD. Clin. Chem. 2009 Apr 9.*
9. *Application of LC/MS and related techniques to high-throughput drug discovery. Espada A, Molina-Martin M, Dage J, Kuo MS. Drug Discov. Today. 2008;13(9-10):417.*
10. *Quantitative analysis of pharmaceuticals in biological fluids using high-performance liquid chromatography coupled to mass spectrometry: a review. Plumb RS, Dear GJ, Mallett DN, Higton DM, Pleasance S, Biddlecombe RA. Xenobiotica. 2001;31 (8-9):599.*
11. *The determination of nicotinic acid in plasma by mixed-mode liquid chromatography-tandem mass spectrometry following ion exchange solid phase extraction. Mallett DN, Dayal S, Dear GJ, Pateman AJ. J. Pharm. Biomed. Anal. 2006;41(2):510*
12. *Automated SPE and Tandem Mass Spectrometry without HPLC columns for Quantifying Drugs at the Picogramme Level. Bowers, G. D.; Clegg, C. P.; Hughes, S. C.; Harker, A. J.; Lambert, S. LC-GC 1997, 15, 48*
13. *Generic serial and parallel on-line direct-injection using turbulent flow liquid chromatography/tandem mass spectrometry. Grant RP, Cameron C, Mackenzie-McMurter S. Rapid Commun. Mass Spectrom. 2002;16(18):1785*
14. *Generic method for on-line extraction of drug substances in the presence of biological matrices using turbulent flow chromatography. Herman JL. Rapid Commun. Mass Spectrom. 2002;16(5):421*
15. *Parallel ultra-high flow rate liquid chromatography with mass spectrometric detection using a multiplex electrospray source for direct, sensitive determination of pharmaceuticals in plasma at extremely high throughput. Bayliss MK, Little D, Mallett DN, Plumb RS. Rapid Commun. Mass Spectrom. 2000;14(21):2039*
16. *LC-MS Development strategies for quantitative bioanalysis. Jemal M, Xia YQ. Curr. Drug Metab. 2006;7(5):491.*
17. *Direct analysis of pharmaceutical compounds in human plasma with chromatographic resolution using an alkyl-bonded silica rod column. Plumb R, Dear G, Mallett D, Ayrton J. Rapid Commun. Mass Spectrom. 2001;15(12):986.*
18. *Ultrahigh-pressure reversed-phase liquid chromatography in packed capillary columns. MacNair JE, Lewis KC, Jorgenson JW. Anal. Chem. 1997 15;69(6):983.*
19. *Fast Liquid Chromatography and Liquid Chromatography/Mass Spectrometry Analysis of Antibiotics Using Rapid Resolution HT HPLC Columns with Sub Two-Micron (1.8 um) Particles, Agilent Application Note 5989-0025EN, Sept 2003*
20. *Peak capacity in gradient ultra performance liquid chromatography (UPLC). Wren SA. J. Pharm. Biomed. Anal. 2005;38(2):337.*
21. *Evaluation of ultra-performance liquid chromatography in the bioanalysis of small molecule drug candidates in plasma. Goodwin L, White SA, Spooner N. J. Chromatogr. Sci. 2007;45(6):298.*
22. *Orthogonal extraction/chromatography and UPLC, two powerful new techniques for bioanalytical quantitation of desloratadine and 3-hydroxydesloratadine at 25 pg/mL. Shen JX, Wang H, Tadros S, Hayes RN. J. Pharm. Biomed. Anal. 2006;40(3):689*
23. *UHPLC – Why all the hype? Houghton R and Grace PB, Chromatography Today Feb 2008, 5.*
24. *Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. Chambers E, Wagrowski-Diehl DM, Lu Z, Mazzeo JR. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2007;852(1-2):22.*
25. *Which human metabolites have we MIST? Retrospective analysis, practical aspects, and perspectives for metabolite identification and quantification in pharmaceutical development. Leclercq L, Cuyckens F, Mannens GS, de Vries R, Timmerman P, Evans DC. Chem. Res. Toxicol. 2009;22(2):280.*