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Detection and identification of suspected materials caused death in deceased specimens by high resolution LC/MS

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ABSTRACT

A 37-year-old woman committed murdered by oral ingestion of hair dye or overdose of some medications were available. A hair dye and many over-the-counter drugs misused in suicide/homicide purpose are uncommon in Libyan society; Women deceased specimens suspected to contain PPD and/or other medication were collected, LC-ESI-MS analysis were carried out to identify the individual substances present in post-mortem specimens. The present work describes an analytical method for determination of lower traces of para-phenylenediamine (PPD) as a major life threatening compound in hair dye as well as paracetamol in post-mortem biological fluids, this procedure involves the protein crash extraction for urine, blood and stomach contents. As well as colour tests as a routine testing were conducted on urine sample, for paracetamol, and it was detected in urine specimen by the Cresol-ammonia colour test.

Key words: Para-phenylenediamine (PPD), LC/MS, APAP, PAP.

INTRODUCTION

Toxicological investigations are generally carried out to study the poisonous effect of signal substance, but the death by poisonous substances can happen in a variety of ways, suicidal ingestion of some substances could cause poisoning such as hair dye, drugs such way of them. This requires specialized tests and the laboratory should alert to its possible usage or involvement in the death when requests for toxicology testing are submitted. Recently HPLC-MS (commonly known as LC/MS) have become increasingly popular tools in forensic toxicological laboratories due to the sensitivity, rapid analysis and simple sample pre-treatment requirements [1].

On the other hand, circumstantial evidences as well as the post-mortem signs were much more helpful in detecting the specific poison in poisoned cases, especially in case of poisons which leave characteristic appearances. In the present case, hair dye around the victim was found, and when her body was autopsied in the morgue on the same day, stained with dark and non-washable colour on the dorsum of the tongue as well as on the stomach mucosa was detected, the lungs were found markedly congested with evident pulmonary oedema, thus circumstantial evidence and post-mortem signs were correlated and then 100 cm2 of dark suspicious stomach liquid content collected for PPD analysis. These previous signs were reported in many cases of PPD toxicity. Thus far, a number of studies have indicated that the majority of the PPD poisoning cases were intentional was ingested with suicidal intent of hair dye (78.1%) [2,3], although many countries considered PPD to be dangerous and banned its used in composition of hair dyes [4].

Para-phenylenediamine (PPD) is a common chromophoric ingredient in oxidative hair-dyes. In some African countries like Sudan, Egypt and Morocco, despite a low risk of intoxication when used in due form, there are

numerous cases of acute intoxication in those countries every year. For instance, at the ENT Hospital - Khartoum (Sudan) alone, more than 300 cases are reported every year and approximately 10% of poisoned cases were fatal [5]. The study by Stambouli (2004) offers probably the most comprehensive empirical analysis of PPD carried out for identifying para-phenylenediamine in post-mortem blood and stomach contents from poisoned case ingested this product using thin-layer chromatography, ultraviolet spectrometry, GC-MS and HPLC-UV [6]. Bellimam et al was developed an analytical method for determination of lower traces of para-phenylenediamine in post-mortem biological fluids. This procedure involves deproteneization or hydrolysis followed by liquid-liquid extraction and derivatization with trifluoroacetic anhydride, the extract was then analysed by gas chromatography/ion trap mass spectrometry [7].

In addition, the most tedious aspect of detecting and identifying poisonous substances in biological samples is the extraction process which required isolating them from the materials submitted for the analysis. In this study, simple sample pre-treatment was used as protein crash extraction and then direct injection carried out. High sensitivity and resolution LC/MS instrument was provided to detect the low trace amount of unknown poisonous substances using manual process of Xcalibur software.

MATERIALS AND METHODS

2.1 Chemicals

All the reagents were analytical reagent grade. HPLC grade acetonitrile was obtained from Fisher Scientific, UK, and Formic acid (98%) was obtained from BDH, (DORSET, UK), water was obtained from in-house Milli-Q water purification station.

2.2 Instrumentation

In this study, samples were analysed using Exactiveorbitrap spectrometer operated on –line with UltiMate® 3000 Standard LC systems (DionexCorporation,USA) , combined with orbitrap mass spectrometer , which was obtained from (Thermofisher, Hemel Hempstead, UK) , equipped with an electrospray ionization (ESI) source system, and a data system (Xcalibur Version 2.0). The interface was adjusted to the following conditions: ion mode was positive and negative switch mode; spray voltage was 4.5 kV; capillary temperature was 250 °C; sheath gas flow rate (nitrogen) was 45; auxiliary gas flow rate (nitrogen) was 15 (units not specified by the manufacturer). The full-scan mass spectrum to obtain the protonated molecules $[M + H]^+$ of each metabolite was collected in the mass range from m/z 50 to 1200.

The LC conditions were as follows: The column fitted with ZIC-HILIC Column (5µm, 150×4.6mm; SeQuant, Darmstadt, Germany) and guard column (HiChrom Limited, Reading, UK) were used for all analysis. The mobile phase consisted of Acetonitrile /water/formic acid, at a flow rate of 0.3 mL min^{-1}. The elution was carried out by binary gradient mode in which Solvent A was 0.1% v/v formic acid/water; and solvent B was 0.1% v/v formic acid/acetonitrile, with an injection volume of 10 µL. The gradient programme was as follows: 20% A (0 min) to 50% A (at 12 min), to 50% A (at 26 min), to 80% A (at 28 min), to 80% A (at 36 min.), to 20% A (at 37 min) and finally to 20% A at 46 min.

2.3 Protein Crash Method for Extraction of outpost materials

The crash plates are attached to a vacuum manifold and protein was crashed from the samples as following steps: The samples were thawed, and then 0.2 mL of sample mixed with 0.8 mL acetonitrile and the two liquids are allowed to mix slowly while the protein precipitates. The acetonitrile was added first and subsequent filtration through a BiotageIsolute® PPT+ protein precipitation plate (Biotage Limited, Sweden). The filtrate was collected and analysed by LC–MS [8].

2.4 Presumptive test of Paracetamol

Presumptive tests, based on a colour reaction, are known as fast screening procedures that can be designed to indicate that the drug classes are either present or absent in the test sample, the direct test described by Clarke was modified and used, 0.5 ml of urine add 0.5 of hydrochloric acid and heat for 1 hour at 100 ◦C; to drop of mixture, add 10 ml of water, and 1 ml of 1% phenol, and 2N sodium hydroxide, a blue colour appeared to indicate that paracetamol or its metabolites were present [9].

RESULTS AND DISCUSSION

The sufficient accuracy has been achieved by employing liquid chromatographic separation in conjunction with orbitrap mass spectrometer technology, which capable of mass accuracies better than 2 ppm. This high level of mass

resolution makes it possible to determine the empirical formula of unknown analytes in full scan mode across the mass range of the instrument.

PPD was suspected in this case, physical objects and their disposition, as well as circumstantial references connecting these data to candidate this substance as life threatening and might cause death. Urine is the most common sample used for drug testing in the workplace, but it is not always available for post-mortem testing. Urine testing results do not directly correlate to drug affects at the time of sample collection because of the time it takes the body to eliminate these drugs or their metabolites (the body's breakdown products) in the urine. Its usefulness lies in the fact that the presence of a substance in the urine is a sign that the substance had been in the blood at an earlier time (usually within a few days) and had been somewhat processed (detecting these metabolites gives proof that the drug had been ingested). When urine is available, chemical colour tests can also conduct in the presence of drugs of abuse. Because drugs and poisons can often be ingested, stomach contents can also provide important investigative clues. High concentrations of drugs or poisons may be detected, depending on how much time elapsed between ingestion and death. In many cases of acute poisoning, undissolved capsules or tablets may be discovered, allowing relatively simple drug or poison identification. The total amount of a drug or poison present in the stomach is more important than its concentration in the blood because it has not been processed by the body yet.

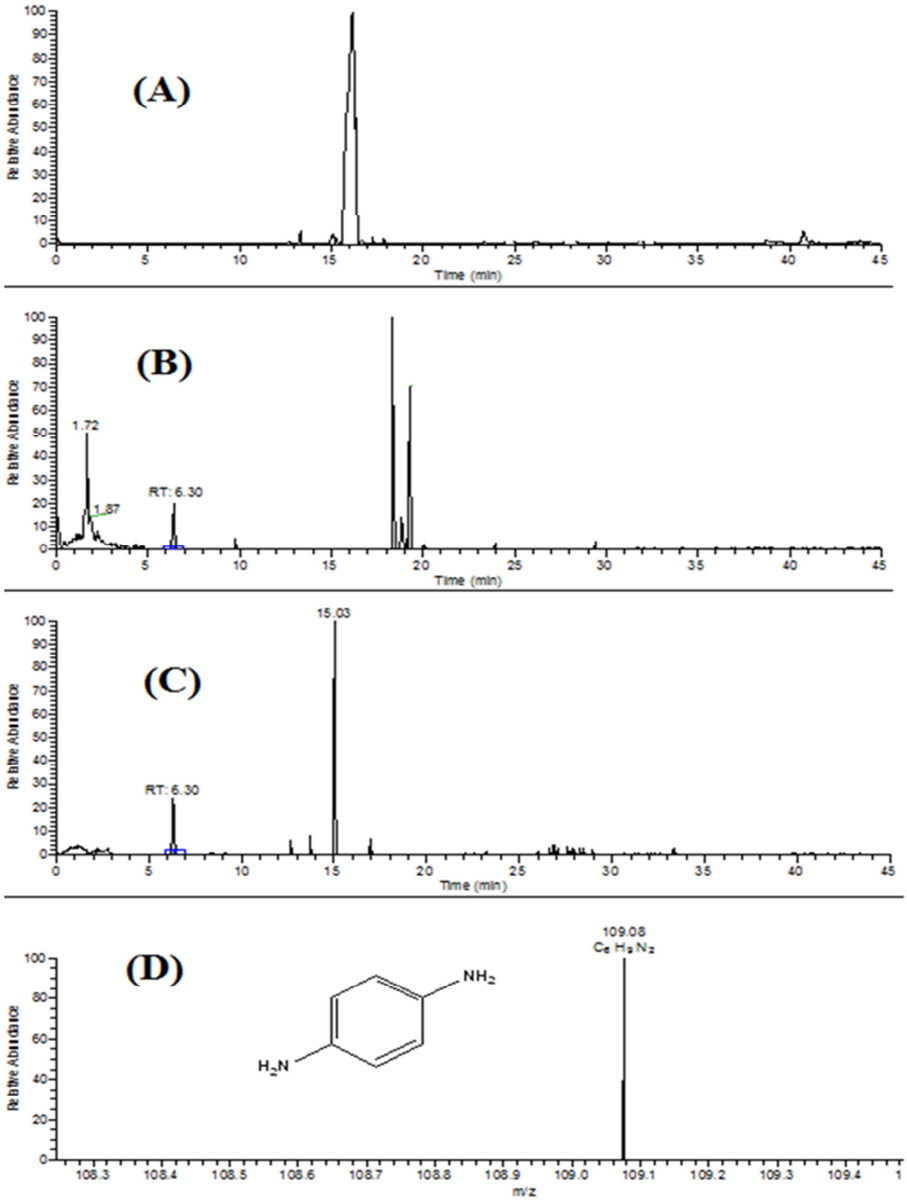

Figure 1. Selected ion chromatograms from LC-MS analysis and mass spectra of PPD in (A) urine(absent)(B) Blood (present)(C) Stomach content(present), selected ion chromatograms illustrate the M^+ ion at m/z =109.08 (D) the mass spectra of the 109.08 M^+ ion identify PPD

Whereas a Liquid chromatography instrumentation was utilised, plasma or serum contains is composed of about 10% w/v protein, thus have to be removed it from the specimens for good chromatographic peak shape and avoid precipitate out of the chromatography column and then caused column blockage. That's why protein crash used in this study, in addition, using Acetonitrile was found much better at removing protein than methanol, and less better was Acetone due to ketone which is more reactive and might be reacting with primary amine in PPD [10].

Specimens were analysed using high resolution LC/MS (university of Strathclyde, SIPBS, UK), after screening the results of three specimens, to detect PPD, sedative, organophosphorus compounds and tranquillizers. The data processed manually and PPD is detected in blood and stomach content specimens, as well as its metabolites N-mono-acetylated (MAPPD) and N, N'-diacetylated PPD (DAPPD) were present and detected in blood and stomach content specimens only in trace amounts and in the urine specimen were absent. Although reported in previous studies that the major urinary metabolites were concluded to be N-mono-acetylated and N,N'-diacetylated PPD [11], the absence of these metabolites could evidently the PPD does not excrete in urine by renal excretion process.

The LC/MS instrument of high resolution could be determined a mass peak with sufficient accuracy, figure 1 showed selected ion chromatogram (SIC) trace obtained from the analysis of PPD in blood, urine and stomach content specimens illustrate the M^+ ion at m/z 109.10, PPD peak was absent at urine specimen (A), The chromatograms (B, C) represent [M+1] peaks of PPD (t_R= 6.3, m/z = 109.08).

The identification of an unknown compound is a challenge for analytical chemistry in forensic analysis, in this study a procedure was developed for the identification of unknowns by using a large accurate mass. The theoretical exact masses of the protonated compounds $[M+H]^+$were calculated in the Xcalibur software based on the molecular formula resulting in an accurate mass. It was manually utilised to detect some other over-the counter medication might cause intoxication, LC-ESI-MS orbitrap was able to detect and locate low intensity peaks.

Figure 2 showed peaks eluting at 4.24, 5.46 and 7.02 mins in urine specimen were subsequently identified as paracetamol, and its hydroxylated and glucuronide metabolites respectively. Figure 3 shows PAP (p- aminophenol) which was detected in urine specimen as well, but did not detect in stomach contents and blood specimens on chemical analysis. PAP was identified as a metabolite of APAP (Paracetamol) which is also detected in urine sample [12]. Moreover APAP metabolite which known as intermediate product NAPQI is detected in urine sample only.

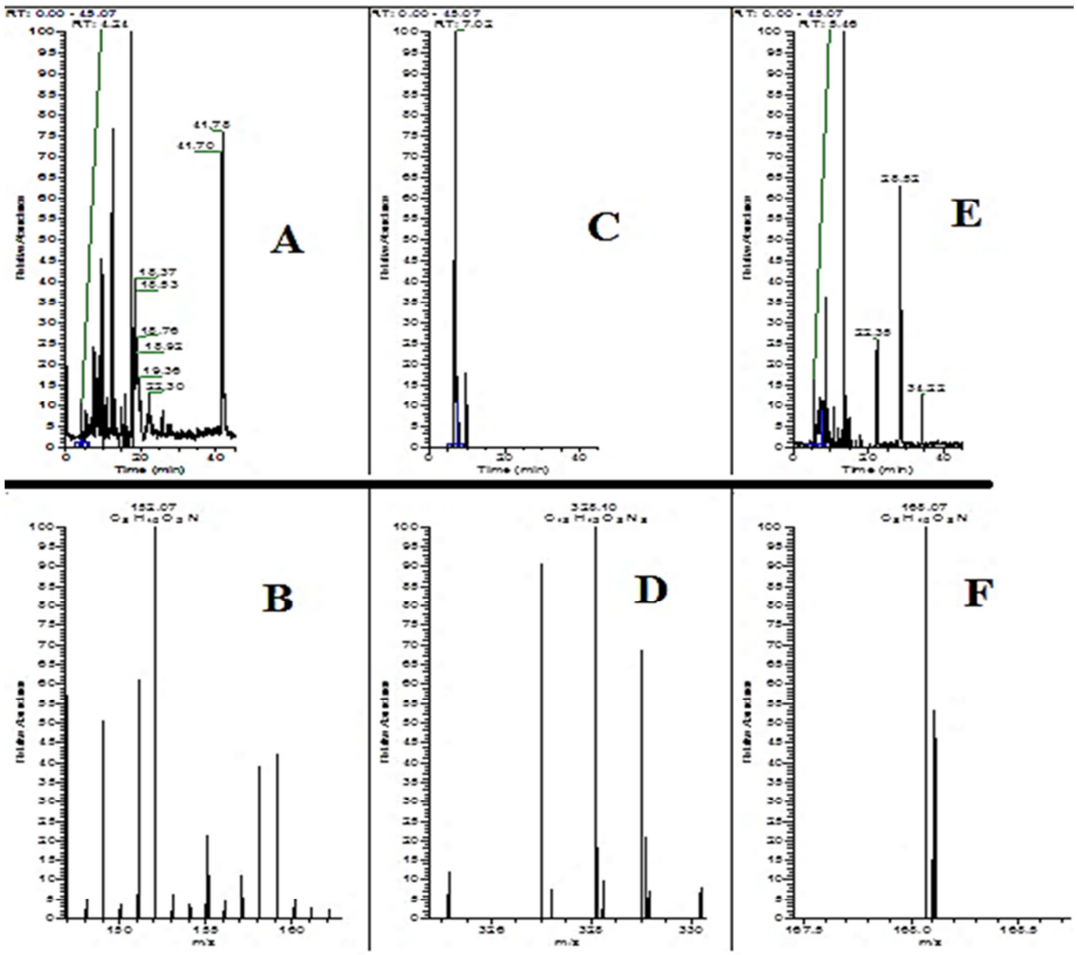

Figure 2.Selected ion chromatograms from LC-MS analysis and mass spectra of Paracetamol and its metabolites in urine sample. (A) selected ion chromatograms illustrate the M+ ion of Paracetamol at m/z =152.07(B) the mass spectra of the 152.07 M+ ion identify Paracetamol compound; (C) selected ion chromatograms illustrate the M+ ion at m/z =328.10 of glucuronide metabolite(D) the mass spectra of the 328.10 M+ ion identify the glucuronide paracetamol ;(E) selected ion chromatograms illustrate the M+ ion at m/z =168.07 of hydroxylated metabolite (F) the mass spectra of the 168.07 M+ ion identify the hydroxylated metabolite

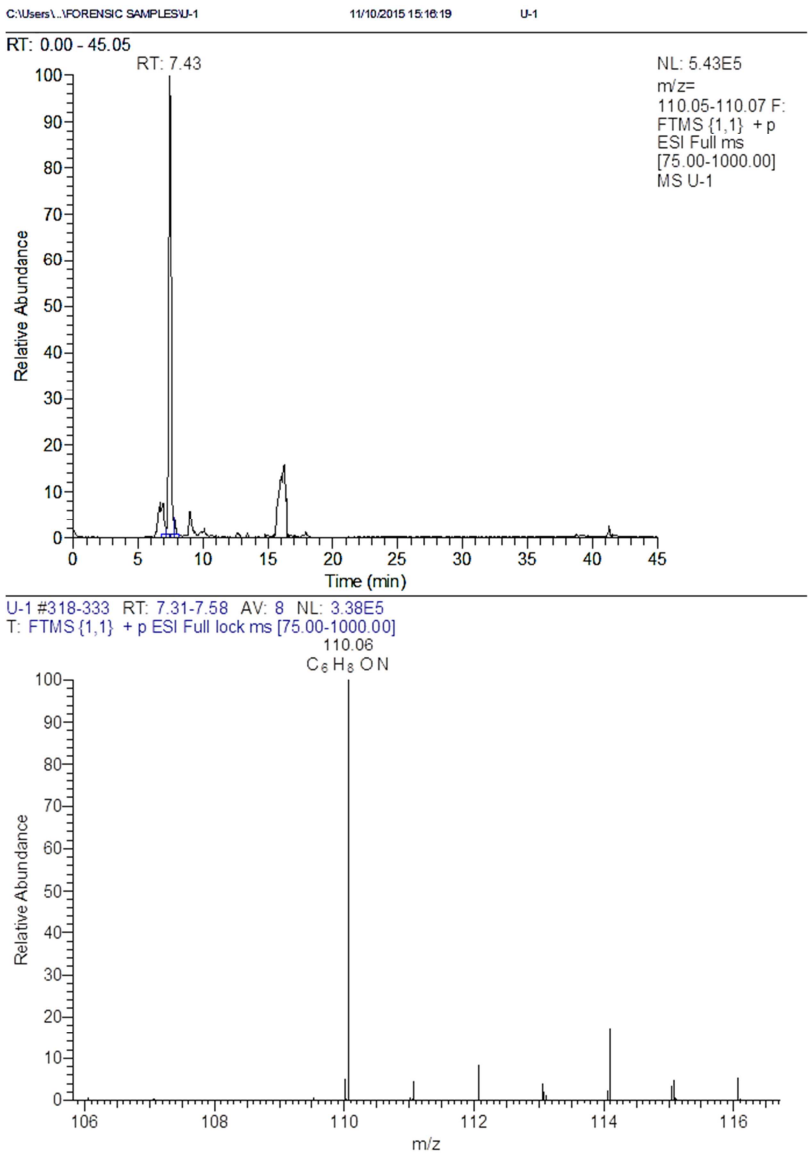

Figure 3.LC-MS chromatogram and Mass spectrum of PAP (p- aminophenol) of paracetamol

As shown above that accurate mass alone may not be enough to correctly identify an unknown compound and more parameters may be needed for identification. Thus , Xcalibur contains the RDB column displays the ring and double-bond equivalents for the unknown compound detected as well as mass tolerance column displays how close a measured mass must be to the locked mass to be considered the same mass, hence these tool much helpful for elucidation the unknown substances, and used in this study to identify the PPD and paracetamol in raw data obtained from LC/MS.

On the other hand, urine specimen is preferred for first –line of systematic toxicological analysis, which is consider as combinations of analytical methods aimed at detecting an unknown foreign substance in biological fluid [13]. Therefore, chemical colour tests carried out to combine with LC-MS analysis of paracetamol and the blue colour was appeared to indicate that urine sample contains paracetamol and its metabolites.

As a final point, to confirm the identity of these compounds further analysis is required to use standard reference compounds. Nevertheless, the combination of colour test with a large accurate mass has proven useful, because the Cresol-ammonia colour test is very specific test for detection Paracetamol and its metabolites was described by Clarke, but *o*-cresol and 2N ammonium hydroxide were replaced by 1% phenol and sodium hydroxide respectively, and found that this test is sensitive and also could detect the conjugated metabolites of paracetamol in urine specimen [14].

CONCLUSION

A dramatic example of the improvement in detection capability afforded by LC-MS orbitrp instrument is illustrated in case studied in this work, detection of an unknown analytes in specimens of urine, blood and stomach content taken post-mortem from a deceased woman with unknown cause of death. LC-MS spectra of extracted specimens show unknown peaks, the instrument is able to assign mass values with sufficient accuracy to propose an empirical formula for the primary

$[M+H]^+$ion of analysts.
Our results show that intact PPD or its metabolites can be detected in postmortem specimens by a dedicated high resolution/high accuracy LC-ESI-MS method, PPD was observed in specimens of blood, and stomach content, but Paracetamol and its hydroxylated and glucuronide metabolites were observed only in urine specimen in trace amounts. A PAP metabolite of paracetamol was detected in urine specimen with reasonable amount. The dynamic range of the instrument was sufficient to show all these compounds and their metabolites.

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REFERENCES

[1] M. Wood, M. Laloup, N. Samyn, M. del Mar Ramirez Fernandez, E. A. de Bruijn, R. A. A. Maes and G. De Boeck, *Journal of Chromatography A* **2006**, 1130, 3-15.
[2] S. C. Chaudhary, K. K. Sawlani and K. Singh, *Niger J ClinPract* **2013**, 16, 258-259.
[3] S. I. Filali A, Ottaviano V, Furnari C, Corradini D, Soulayman R., *Afr J Trad CAM*. **2006**.
[4] K. V. M. M. D. Radhika , M. Sreenivasulu , Y.Srikanth Reddy , T. S. Karthik *JBST* **2012**, Volume 2012; 3 492–497
[5] G. P. Hooff, N. A. van Huizen, R. J. W. Meesters, E. E. Zijlstra, M. Abdelraheem, W. Abdelraheem, M. Hamdouk, J. Lindemans and T. M. Luider, *PLoS ONE* **2011**, 6, e22191.
[6] A. Stambouli, M. A. Bellimam, N. El Karni, T. Bouayoun and A. El Bouri, *Forensic SciInt* **2004**, 2, S87-92.
[7] M. A. Bellimam, A. Stambouli, N. El Karni, T. Bouayoun and A. El Bouri, *ActaClinBelg* **2006**, 1, 41-47.
[8] M. Hayward, M. D. Bacolod, Q. P. Han, M. Cajina and Z. Zou in Techniques to Facilitate the Performance of Mass Spectrometry: Sample Preparation, Liquid Chromatography, and Non-Mass-Spectrometric Detection, Vol. John Wiley & Sons, Inc., **2011**, pp. 353-381.
[9] J. Swarbrick, *Journal of Pharmaceutical Sciences* **1987**, 76, 420-421.
[10] C. Polson, P. Sarkar, B. Incledon, V. Raguvaran and R. Grant, J *Chromatogr B AnalytTechnol Biomed Life Sci* **2003**, 785, 263-275.
[11] G. J. Nohynek, J. A. Skare, W. J. Meuling, D. W. Hein, A. T. De Bie and H. Toutain, *Food ChemToxicol* **2004**, 42, 1885-1891.
[12] S. E. McConkey, D. M. Grant and A. E. Cribb, *J Vet Pharmacol Ther* **2009**, 32, 585-595.
[13] P. Marquet in Systematic toxicological analysis with LC-MS, Vol. (Ed. A. Polettini), p. 111.
[14] L. H. Higgins G. in Screening Tests for Common Drugs, Vol. 2 (Ed. C. E. G. C.), the Pharmaceutical Press, London, **1975**, pp. 873-913.