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Protein precipitation for the analysis of a drug cocktail in plasma by LC–ESI–MS

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Abstract

Three protein precipitation (PP) procedures with acetonitrile (ACN), perchloric acid (PA) and trichloroacetic acid (TCA) were investigated for the analysis of a drug cocktail from human plasma samples containing three pharmaceutical compounds and their primary metabolites. For this purpose, a capillary liquid chromatography–electrospray ionisation–mass spectrometry (LC–ESI–MS) method was developed for the simultaneous analysis of the six tested compounds in less than 6 min. Matrix effect was tested for each PP procedure by means of a post-column infusion system. The three PP techniques were found effective in removing proteins from human plasma and were fully compatible with capillary LC–ESI–MS analysis. However, with acid precipitations, low analyte recovery and a high variability, probably due to analyte coprecipitation, were obtained. Finally, ACN was found to be the most effective PP technique with a recovery higher than 80% and CV inferior to 6%.

Keywords: Protein precipitation; Analytes co-precipitation; Capillary LC–ESI–MS; Matrix effect

1. Introduction

Liquid chromatography–mass spectrometry (LC–MS) is considered as the technique of choice for a fast, selective and sensitive analysis of drugs and metabolites in biological fluids. In the last years, miniaturized LC systems have raised renewed interest thanks to a low injection volume, a reduced solvent consumption and an increase in sensitivity [1–4]. Additionally, a low mobile phase flow rate improves LC compatibility with MS detection equipped with electrospray ionisation (ESI) source. Given the miniaturization of the analytical system (i.e. analytical column, tubing, ESI sprayer) and the peak concentration, sample preparation remains also of utmost importance to ensure good analytical results and system safety. Indeed, biological matrices (i.e. serum and plasma) are complex mixtures containing numerous endogenous components such as proteins, salts or lipids which can interfere with analytes during the separation and detection processes. Among these interferences, pro-

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teins which are present in a large amount in plasma or serum can be irreversibly adsorbed onto the chromatographic support, which causes the deterioration of separation efficiency and a rapid column clogging. A common procedure to remove proteins in biological samples by protein precipitation (PP) is to add a reagent (organic solvent, acids or salts) [5–7]. Up to 98% of the proteins in human plasma can thus be eliminated with an appropriate additive [1,8]. Protein precipitation procedures are considered as fast, easy to handle biological samples and can be applied to a wide range of analytes. PP techniques can be used for blood samples (i.e. serum, plasma) as sample cleanup procedures as well as a pre-treatment, coupled to other extraction techniques. After PP, the isolation of filtrate is generally performed by centrifugation or filtration. Then, a small amount of the supernatant can be directly injected with or without pH adjustment or concentrated by solvent evaporation.

It is noteworthy that the recent automation of this technique with a 96-well format has widely contributed to its success [9–15]. However, PP procedures suffer from a low selectivity, low analyte recovery or alteration of the MS compound response by co-elution of endogenous material. As an example, with PP techniques, analyte recovery is inferior to 60% [16,17]. Dawidowicz et al. demonstrated that the poor recovery of propofol was due to an analyte coprecipitation with plasma proteins which mainly depended on PP procedure and plasma composition [16]. The effect of residual endogenous components after PP on electrospray ionisation (ESI) MS signal has been described [8,18,19]. Generally, analyte suppression induces lack of sensitivity, of trueness and precision. Therefore, these limitations must be taken into account during the development of an analytical method using PP procedures.

In this work, three different PP procedures with acetonitrile (ACN), perchloric acid (PA) and trichloroacetic acid (TCA) were investigated for the analysis of a drug cocktail containing three pharmaceutical compounds and their primary metabolites, namely methadone (MTD) and ethylidene-1,5-dimethyl-1,3-diphenylpyrrolidine (EDDP), fluoxetine (FLX) and norfluoxetine (NFLX), flunitrazepam (FLZ) and norflunitrazepam (NFLZ) from human plasma samples. A rapid analysis was carried out by capillary LC–ESI–MS.

2. Experimental

2.1. Chemicals

Fluoxetine and methylfluoxetine hydrochlorides (MFLX) were a gift of Humann Pharma (Nuremberg, Germany). Norfluoxetine, perchloric acid, trichloroacetic acid and ammonium formate were purchased from Sigma (Buchs, Switzerland). Methadone hydrochloride was obtained from Hanseler AG (Herisau, Switzerland) and 2-ethylidene-1,5-dimethyl-1,3-diphenylpyrrolidine perchlorate from Radian International (Austin, TX, USA). Flunitrazepam was obtained from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Norflunitrazepam was kindly supplied by Dr. C. Staub of the Institut Universitaire de Médecine Légale (Geneva, Switzerland). Acetonitrile and formic acid were obtained from SDS (Peypin, France) and water was provided by a Milli-Q Gradient A10 water purifier system from Millipore (Bedford, MA, USA).

Human blank plasma were obtained from the Centre de Transfusion of Geneva Hospital (Geneva, Switzerland).

2.2. Sample preparation

2.2.1. Solutions of the drug cocktail

Stock solutions of FLX, NFLX, MTD, EDDP, FLZ and NFLZ were prepared in a mixture of ACN:water (1:1, v/v) each at a concentration of 1000 $\mu g\,mL^{-1}$. A solution of the drug cocktail at 10 $\mu g\,mL^{-1}$ was obtained by successive dilution of the stock solution and was used to spike blank human plasma.

Two solutions of the drug cocktail at 500 $ng\,mL^{-1}$ and at 2 $\mu g\,mL^{-1}$ were prepared by successive dilutions of the stock solution in water and in the mobile phase, respectively.

MFLX was selected as internal standard (IS). Stock solution of MFLX at 1000 $\mu g\,mL^{-1}$ were prepared in a mixture of ACN:water (1:1, v/v). A solution of 10 $\mu g\,mL^{-1}$ of methylfluoxetine in water was prepared by successive dilution of the stock solution.

2.2.2. Protein precipitant solutions

Acid solutions of PA 6% (v/v) and TCA at 10% (v/v) were prepared by appropriate dilution of concentrated acids with water. Acid solutions and ACN were

spiked with the IS solution to obtain a concentration of 500 ng mL^{-1} of IS.

2.2.3. Protein precipitation procedures

Human blank plasma were spiked with the drug cocktail solution at 10 μg mL^{-1} and were vortex mixed. According to the nature of the protein precipitant (ACN and acids), two procedures can be discerned.

2.2.3.1. Procedure with acid precipitant. 200 μL of acid solution containing 500 ng mL^{-1} of IS was added to 200 μL of spiked plasma. The latter was vortex mixed and centrifuged for 5 min at 6000 × *g*. 100 μL of the supernatant was diluted with 50 μL of ammonium formate 1 M to obtain a pH of the sample of ca. 3.6 before injection in the capillary LC system.

The same procedure was used to treat blank plasma and an aqueous solution with the drug cocktail at 500 ng mL^{-1}.

2.2.3.2. Procedure with acetonitrile. 400 μL of acetonitrile containing the IS was added to 200 μL of spiked plasma. After vortex mixing, the sample was centrifuged for 5 min at 6000 × *g*. 100 μL of supernatant was evaporated to dryness under a stream of nitrogen at 37 °C. The residue was reconstituted with 100 μL of mobile phase constituted of a mixture ACN:water (30:70, v/v) at 0.1% formic acid.

The same procedure was used to treat blank plasma and the aqueous solution of the drug cocktail at 500 ng mL^{-1}.

2.3. Capillary LC–UV-ESI–MS

All experiments were performed on an Agilent 1100 Series capillary LC system from Agilent Technologies (Waldbronn, Germany) including a capillary pump with an electronic flow control and a diode array detector equipped with a cell of 500 nL and 10 mm path-length. The Chemstation software suite (Agilent Technologies) was used for instrument control, data acquisition and data handling. The MS detection was carried out with an Agilent Series 1100 MSD single quadripole (Agilent Technologies) equipped with an orthogonal electrospray micro-nebulizer. All analyses were performed on a prototype column Zorbax SB-C18, 50 × 0.3 mm i.d., 3.5 μm provided by Agilent Technologies. The post-column infusion system was carried out with a Harvard Model 22 syringe pump (South Natick, MA, USA).

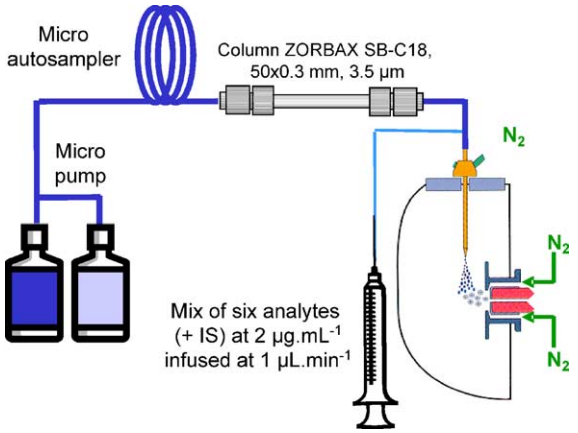

Fig. 1. Post-column infusion system.

2.3.1. Analysis of plasma samples

To determine analyte recovery, 200 nL of prepared plasma was injected. The complete separation of the six analytes was obtained with a mobile phase constituted of a mixture of ACN:water (35:65, v/v) in the presence of 0.1% formic acid at a flow rate of 7 μL min^{-1}.

To evaluate the matrix effect, a post-column infusion system was used (Fig. 1). 200 nL of blank plasma treated by the three precipitants was injected on the capillary LC–ESI–MS while a post-column syringe infused at 1 μL min^{-1} a solution of the drug cocktail at 2 μg mL^{-1} in the mobile phase.

The detection was carried out in the single ion monitoring (SIM) mode. The drying gas (nitrogen) temperature was set at 250 °C with a flow rate of 3 L min^{-1} and the electrospray voltage at 3000 V (positive ionisation mode). The pressure of nebulizing gas (nitrogen) was set at 10 psi (1 psi = 6894.76 Pa). Seven molecular ions were detected with a skimmer voltage optimized for each ion: 160 V for ions 314 (FLZ), 300 (NFLZ) and 278 (EDDP), 100 V for ions 310 (MTD and FLX) and 324 (IS), 80 V for ion 296 (NFLX).

2.3.2. Analysis of the test mixture

100 μL of a test mixture (phenol, naphthalene and anthracene) was injected on the prototype Zorbax col-

umn which was eluted with a mobile phase containing ACN:water (80:20, v/v) at a flow rate of 4 μL min^{-1}. The detection was carried out with UV diode array detector at 254 nm.

3. Results and discussion

PP is usually performed by adding an organic solvent, an acid or a salt to alter the solubility of proteins in the sample. Several phenomena occur; the mechanisms are described in detail elsewhere [6,20]. However, the precipitant effect on proteins can be summarized as below:

- An organic solvent such as methanol, ethanol, acetonitrile and acetone leads to a decrease of the solution dielectric constant inducing protein precipitation.
- At a pH value lower than the protein isoelectric point, acid reagents interact with the positively charged amine group of proteins to form an insoluble salt.
- The addition of a salt such as ammonium sulphate induces, at high concentration, a salting-out effect and proteins precipitate from solution.

The capacity of different precipitants to eliminate protein in plasma samples was evaluated by Blanchard [1] and more recently by Polson et al. [8]. In 1981, Blanchard evaluated the efficiency of twelve PP techniques for removing protein in human pooled plasma [1]. Only a small amount of diluted TCA and PA (1 volume of precipitant for 1 volume of plasma, 1:1, v/v) enabled to precipitate more than 98% of protein while the same efficiency was obtained with a higher ratio of ACN to plasma volume [1]. This procedure was confirmed by Polson et al. [8] who demonstrated that ACN (2:1, v/v) and TCA (1:1, v/v) were found to be the best techniques for all the studied plasma samples, including human plasma.

However, if PP is recognized as a generic and efficient procedure for protein removal, it is also considered as a non-selective technique; furthermore, low analyte recovery or/and lack of selectivity were frequently observed.

The aim of this work is to investigate the commonly used protein precipitation procedures for the analysis of a drug cocktail containing three pharmaceutical compounds and their primary metabolites by capillary LC–ESI–MS. Given the high protein removal efficiency demonstrated by Blanchard [1] and Polson et al. [8], PP with ACN, TCA and PA was selected. The influence of the three procedures on the ESI–MS signal, on analyte recovery and chromatographic performance were studied.

3.1. Matrix effect and optimization of LC separation

As previously mentioned, PP procedures are non selective purification techniques; precipitated plasma samples can contain a high amount of interfering compounds. With UV detection, they can affect the chromatographic signal and with MS detection, interferences can disturb the analyte ionisation process especially with an ESI source. The most common effect observed on ESI–MS is a signal suppression which can alter quantitative and qualitative performances. A matrix effect was already described for the analysis of biological samples precipitated by PP techniques [8,18,21,22]. As an example, Bonfiglio et al. demonstrated ESI–MS suppression at the beginning of the chromatogram after plasma precipitation with ACN [22]. Therefore, under these conditions, a sufficient chromatographic retention of the analyte was required for reproducible and accurate results. Therefore, matrix effect on LC–UV and LC–ESI–MS methods should be carefully determined [21–23].

In order to reduce or eliminate this undesirable matrix effect, several strategies are described in the literature such as a more selective extraction procedure and/or chromatographic separation as well as alternative ionization process (i.e. atmospheric pressure chemical ionization or APCI) [21,23].

In this paper, the matrix effect of the three PP techniques on ESI–MS response was evaluated and a post-column infusion system was employed (Fig. 1) [22]. Six human blank plasma from different origins were each prepared following the three PP techniques and were injected. Data were recorded in the single ion monitoring (SIM) mode. Examples of MS chromatograms are reported in Fig. 2. A significant signal suppression was observed in ACN precipitation for the first 2 min of the chromatogram with an octadecyl column, which was in agreement with Polson et al. [8]. The same behaviour was observed for acid procedures and for each tested plasma. These

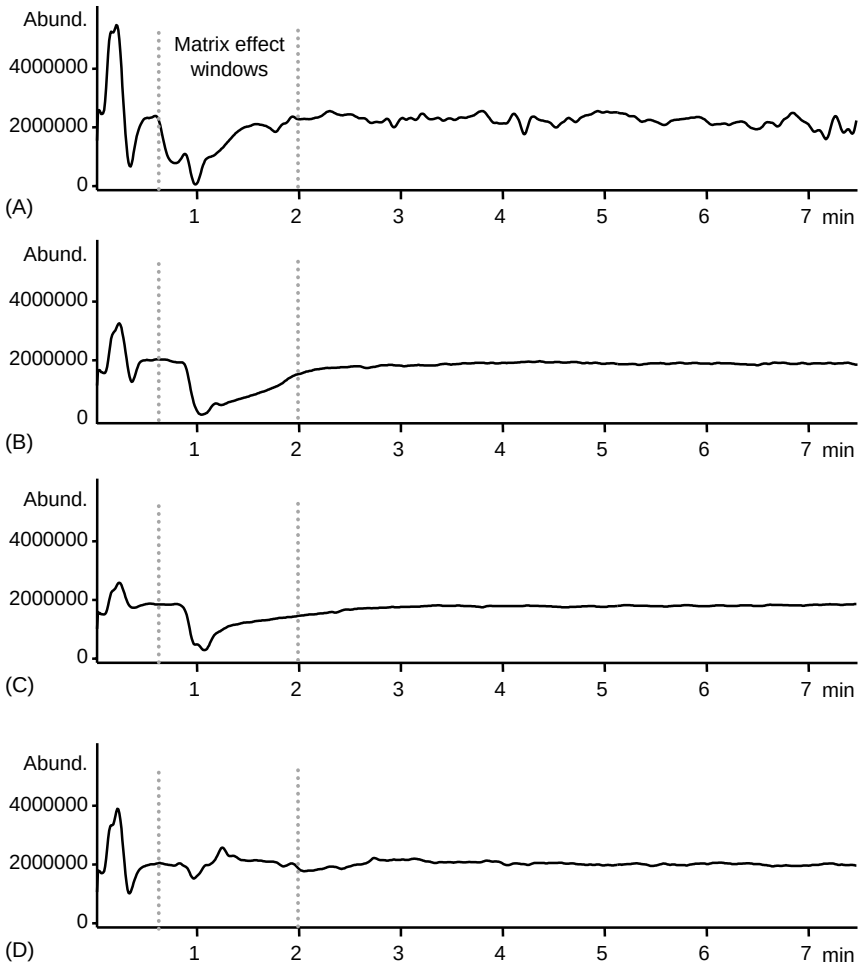

Fig. 2. Total ion chromatograms obtained for the analysis of human blank plasma treated following the three PP techniques. (A) plasma extracted by ACN; (B) plasma extracted by PA; (C) plasma extracted by TCA; (D) mobile phase injection (reference).

results suggest that the interferences in the sample after each precipitation technique were mainly polar. Consequently, special attention has to be given to the first 2 min of the run where polar metabolites such as EDDP can be eluted from the capillary column.

Another requirement must be taken into account during the development of a chromatographic separation. Indeed, among the compounds of interest, two molecules (FLX and MTD) were isobaric compounds and, despite the high selectivity of the single MS system, a chromatographic resolution between these compounds remains necessary. A mobile phase constituted of ACN:water (30:70, v/v) in the presence of 0.1% formic acid was established as suitable. Under optimized conditions, a resolution of 1.86 between MTD and FLX was obtained while the more polar analyte EDDP exhibited a retention time of 2.6 min. NFLZ, NFLX, MTD, FLX, FLZ and MFLX were eluted at 2.8, 3.7, 3.8, 4.3, 4.4 and 4.9 min, respectively. The total analysis time was less than 6 min (Fig. 3).

3.2. Analyte recovery

A sample cleanup procedure has to fulfill different criteria such as a good repeatability and a sufficient recovery. In order to evaluate the potential of the three PP techniques, six human plasma from different origins were spiked with the drug cocktail (final con-

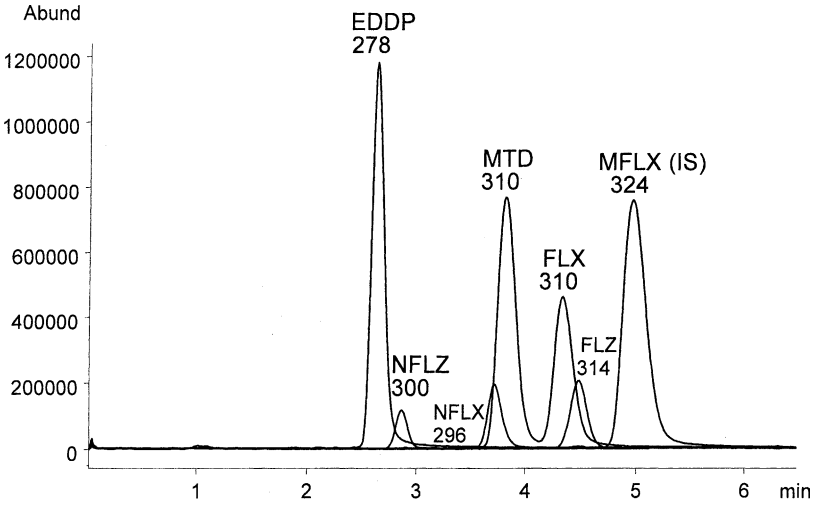

Fig. 3. ESI–MS chromatogram of an aqueous solution containing the drug cocktail at 500 ng mL^{-1} and analysed by capillary LC–ESI–MS. Conditions are described in Section 2.

centration 500 ng mL^{-1}) and injected into the capillary LC–ESI–MS system. A typical chromatogram of a plasma precipitated with ACN is presented in Fig. 4. Recoveries for each analyte were determined by injecting a standard aqueous solution of the drug cocktail at 500 ng mL^{-1} treated by the three techniques. Repeatability of each PP procedure was assessed. Preliminary experiments demonstrated that intra-plasma variability (CV obtained with plasma from the same origin prepared six-fold), was always inferior or not significantly different (F test; $\alpha = 0.05$) than inter-plasma variability (data not shown). Therefore, further investigations were conducted with plasma coming from different origins. Results are reported in Table 1. Analyte recoveries between 80 and 120% were obtained for MTD, EDDP, FLZ, FLX, NFLX and MFLX with CV values inferior to 6.0% for acetonitrile precipitation. Only NFLZ showed a recovery too high. With perchloric acid precipitation, recoveries were inferior to 40% for MTD, FLX and their metabolites with CV values superior to 5%. However, FLZ and NFLZ showed higher recoveries with better repeatability (CV inferior to 6%). Plasma precipitated by TCA showed recoveries inferior to 25% for MTD, FLX and their metabolites with CV values superior to 6%. Analyte recoveries of 64 and 65% with CV superior to 6% were observed for FLZ and NFLZ, respectively. Thus, low analyte recovery with PA and TCA could be due to a co-precipitation. This phenomenon was less relevant for neutral compounds (FLZ and NFLZ), while the high recovery observed for NFLZ could not be explained.

Table 1
Analyte recovery and repeatability for the capillary LC–ESI–MS analysis of human plasma with the three PP techniques

PP techniques		MTD	EDDP	FLX	NFLX	FLZ	NFLZ	MFLX (IS)
ACN	Recovery (%)	109	102	83	112	83	133	85
	CV (%)	3.61	4.38	5.3	2.16	4.27	4.14	5.06
PA	Recovery (%)	35	34	20	19	89	136	18
	CV (%)	6.10	6.83	5.84	5.41	2.87	5.28	6.51
TCA	Recovery (%)	17	21	15	9	64	65	9
	CV (%)	12.20	6.78	13.58	13.27	8.86	6.51	13.15

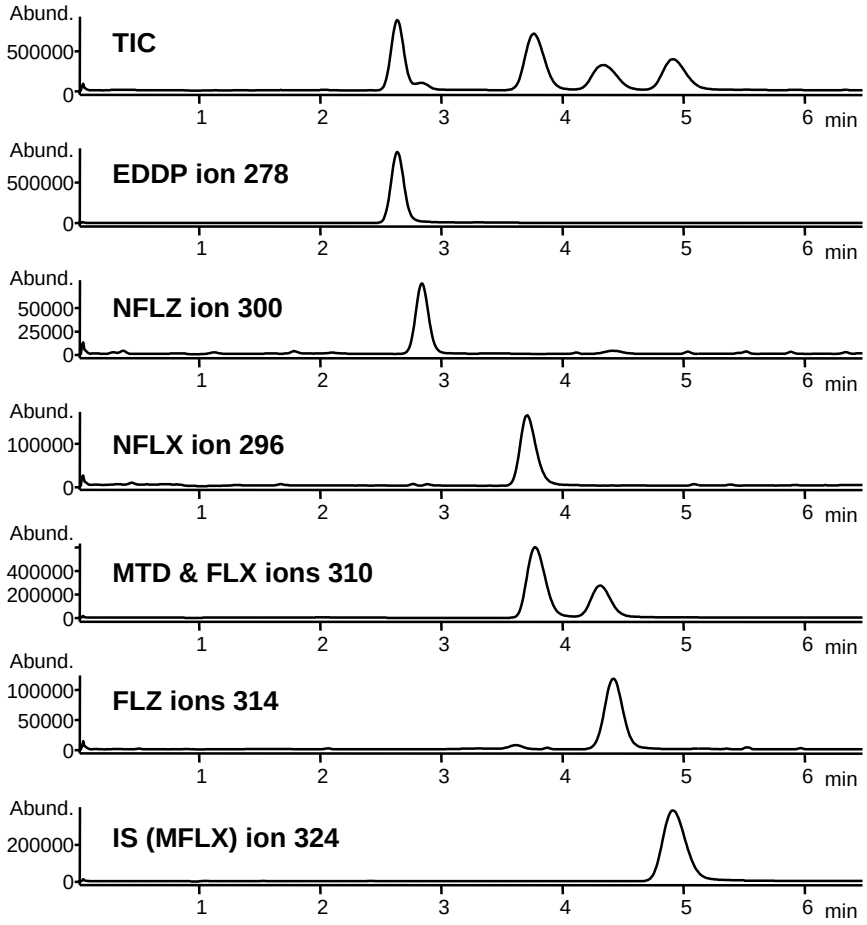

Fig. 4. ESI–MS chromatogram of human plasma spiked at 500 ng mL^{-1} of a drug cocktail and precipitated by ACN. Conditions are described in Section 2.

3.3. Column efficiency test

One-hundred fifty injections of plasma were performed onto the capillary LC–ESI–MS system, corresponding to a total volume of plasma of ca. 15 μL. No pressure increase was observed during all experiments and retention times were held constant. In order to evaluate column performances, a conventional test mixture containing phenol, naphtalene and anthracene was injected before and after the analysis of plasma treated by each PP technique (i.e. after 50 plasma injections). No significant chromatographic deterioration was observed in terms of resolution, retention time and efficiency (data not shown). These results suggest that the three PP techniques lead to extracts sufficiently clean prior capillary LC analysis.

4. Conclusion

Three PP techniques using ACN, PA and TCA were evaluated for the capillary LC–ESI–MS analysis of a drug cocktail in human plasma. In all cases, the presence of endogenous interfering substances in treated plasma led to ESI–MS signal suppression for the first 2 min of the chromatogram. This undesirable effect must be taken into account during chromatographic separation in order to improve method performances. In this context, the separation and detection of six

model compounds and internal standard were obtained in less than 6 min. No significant deterioration of the capillary chromatographic column was demonstrated after the injection of plasma samples precipitated following the three procedures. Among the three selected PP techniques, ACN was the most effective precipitant in terms of analyte recovery and repeatability. With acid PP techniques, analyte recovery was inferior to 50%.

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