Simultaneous determination of six analgesics in human plasma using solid-phase extraction and liquid chromatography

Fernández P¹*, Taboada V¹, Bermejo AM³, Carro AM² and Lorenzo RA²

¹Institute of Forensic Sciences, Forensic Toxicology Service, Faculty of Medicine, C/ San Francisco s/n, 15782 Santiago de Compostela. Spain
²Department of Analytical Chemistry, Faculty of Chemistry, 15782 Santiago de Compostela.

Abstract: An analytical method for the simultaneous determination of paracetamol, salicylic acid, metamizol, tramadol, ibuprofen and diclofenac in plasma, using High Performance Liquid Chromatography with a photodiode array detector (HPLC-PDA) was developed. A solid-phase extraction (SPE) using Oasis HLB cartridges was applied. After conditioning the cartridges with ethyl acetate, methanol and phosphate buffer pH 4, and introducing the plasma sample, the analytes were eluted with ethyl acetate. Then, the eluate was evaporated to dryness, reconstituted in mobile phase, and 30 µL were injected into the chromatograph. The chromatographic separation was performed on an XBridge™ Shield RP18 column (250×4.6 mm id, 5 µm particle size), and the elution was carried out with a mobile phase consisting of acetonitrile, 5mM phosphate buffer pH 6 and Milli-Q water, at a flow of 1 mL/min, in gradient mode. The response of the detector was linear within the concentration range of 0.1-20 µg/mL in human plasma, with coefficients of correlation higher than 0.997. The limits of detection ranged from 7.3 to 30.1 ng/mL. The coefficients of variation were less than 8%, and the recoveries oscillated between 90.1% for diclofenac and 100.2% for salicylic acid. Finally, the method was applied to 16 plasma samples from subjects poisoned with one or more analgesics.

Keywords: Analgesics, plasma, HPLC-PDA, SPE.

Palabras clave: Analgésicos, plasma, HPLC-PDA, SPE.

Introduction

The analgesics are a heterogeneous group of drugs that are frequently prescribed in acute and chronic symptomatic treatments, thus constituting a major cause of morbidity and mortality in the developed countries. The quantification of analgesics in plasma may help to optimize chronic dosing, verify compliance, identify changes in pharmacokinetics and diagnose intoxications. For these reasons, the optimization of reliable, rapid and simple methodologies for the control of these drugs in plasma is required in order to save time and costs, without losing sensitivity and reproducibility of the analytical method. Toxicological analyses are based on sample preparation which is a very important stage because of the complexity of the biological samples and the need to detect low concentrations of the analytes. Sensitivity, selectivity and sample clean-up can be enhanced by commonly employed techniques [1, 2], such as solid-phase extraction, that is used to target compounds and removes excessive impurities, thus helping to reduce the time involved and the solvent volume used [3].

Several chromatographic methods have been described for the determination of analgesics in biological fluids. High performance liquid chromatography (HPLC) is a good alternative for the toxicological study of these polar and thermally unstable compounds in plasma because a derivatization step is not required. Gas chromatography with derivatization step makes the sample preparation laborious and time-consuming. HPLC with mass spectrometry detector [4-7] is highly selective, but the instrumental cost makes it less suitable for toxicological laboratories. In contrast, HPLC with ultraviolet or photodiode array detector is widely available in most analytical laboratories [8-14] due to its lower cost, easier maintenance and adequate selectivity.

The aim of this work was the optimization of a solid-phase extraction and a liquid chromatographic method with photodiode array detector (HPLC-PDA) for the simultaneous determination of paracetamol, salicylic acid (main metabolite of aspirin), metamizol, tramadol, ibuprofen and diclofenac in human plasma. In the extraction procedure pH, conditioning and washing solvents and elution solvents were tested. Finally, this methodology was applied to the analyses of real plasma samples, received in our laboratory for their toxicological interpretation.
Material and methods

Reagents and solutions

Paracetamol, tramadol and diclofenac were obtained from Grünenthal®, Salicylic acid and ibuprofen from Bayer®, and metamizol from Ciclum Farma® laboratories. Potassium dihydrogen phosphate, phosphoric acid, acetic acid, methanol, ethyl acetate, dichloromethane and acetonitrile (LiChrosolv Grade) were purchased from Merck® (Darmstadt, Germany). Ultrapure water was processed using a Milli-Q UV plus system (Millipore, Bedford, MA). The phosphate buffer pH 6 was prepared by solving 0.68 g of KH₂PO₄ in 1 L of Milli-Q water and using KOH 0.5M to achieve the adequate pH. Oasis HLB® cartridges were supplied by Waters® (Milford, MA). Individual stock standard solutions containing 1 mg/mL of each drug were prepared in methanol. Mixture stock standard solutions were prepared by diluting the individual stock solutions to obtain a final concentration of 50 µg/mL and 10 µg/mL in mobile phase.

Plasma samples

Drug-free plasma from the Galician Transfusion Center was used for the preparation of calibration standards of concentration levels 0.1; 0.5; 1.0; 5.0; 10.0 and 20.0 µg/mL. Plasma samples were obtained from patients poisoned with analgesics and stored at 4ºC, unless analysis was delayed, in which case the samples were frozen. All studies were conducted in accordance with the World Medical Association’s “Ethical Principles for Medical Research Involving Human Subjects”.

Sample preparation

The plasma was centrifuged at 4000 rpm for 6 min to separate possible interferences coextracted, that could cause matrix effect. Next, 625 µL of 0.1M phosphate buffer pH 2 were added to 500 µL of plasma containing the analgesics, to obtain a pH 4. The extraction was performed in a Manifold® system, where is possible to process 12 samples simultaneously. The Oasis HLB cartridge was sequentially conditioned with 3 mL of ethyl acetate, 3 mL of methanol and 3 mL of buffer phosphate pH 4. The sample was added and then washed with 0.5 mL of water and 3 mL of ammonium hydroxide 0.05M. The cartridge was dried under vacuum for 5 minutes and the analytes were eluted with 3 mL of ethyl acetate. The extract was evaporated to dryness under a nitrogen stream at 40ºC, and the residue was redissolved in 100 µL of mobile phase. Finally, 30 µL were injected into the HPLC system.

Method

The analyses were performed on a model 2695 liquid chromatograph from Waters® (Milford, MA) connected to a model 996 photodiode array detector also from Waters®. Data were processed using the software Empower Pro®. Chromatographic separation was performed on an XBridge® Shield RP18 stainless steel column (250x4.6 mm id, 5 µm particle size) supplied by Waters®. The mobile phase was a mixture of acetonitrile (A), 5mM phosphate buffer pH 6 (B) and Milli-Q water (C), at a flow of 1 mL/min, in gradient mode: 0-1 min, 10% A and 90% B; 1-9 min, 17% A and 83% B; 9-10 min, 30% A and 70% B; 10-19 min, 52% A and 48% B; 19-21 min, 70% A and 30% C; and 21-22 min 10% A and 90% B.

The method was validated for linearity, limits of detection and quantification, repeatability (intra-day precision), reproducibility (inter-day precision), and accuracy in terms of recovery, according to the recommendations of Peters and Maurer [15] and FDA [16]. The recovery was calculated as the percentage of analyte response after sample workup compared to that of a solution containing the analyte at the theoretical maximum concentration.

Results and discussion

SPE optimization

The SPE was carried out using Oasis HLB cartridges, which exhibit both hydrophilic and lipophilic retention characteristics, and can isolate acidic and basic compounds, whether polar or non polar [17]. Using 3 mL of ethyl acetate, 3 mL of methanol and 3 mL of water for the initial conditioning, and ethyl acetate as the elution solvent, some acid pH (2, 3 and 4) in the conditioning phase and sample loading were tested. Figure 1 shows the best results obtained when pH 4 was used. In the washing stage, acid and basic solvents were tested; for the acid combination, 1 mL of methanol 5% and 3 mL of acetic acid 2% were used, and the basic combination was performed with 0.5 mL of water and 3 mL of ammonium hydroxide 0.3M. Since metamizol was eluted in the acid wash step, the basic washing was selected because the results were good for the most of the analogies.

The drying times of 5, 10 and 15 min were also tested, obtaining the best results with 5 min (Figure 2), reducing the extraction time. In the elution stage, different solvents (methanol, ethyl acetate and mixtures of dichloromethane-ethyl acetate and cyclohexane-ethyl acetate) and their volumes (3, 4 and 5 mL) were tested; the best recoveries for all analogies were obtained with 3 mL of ethyl acetate.

Figure 1. Evaluation of the pH in conditioning phase and sample loading (PAR: Paracetamol; SA: Salicylic acid; MET: Metamizol; TRA: Tramadol; IBU: Ibuprofen; DIC: Diclofenac)

Figure 2. Evaluation of drying time (PAR: Paracetamol; SA: Salicylic acid; MET: Metamizol; TRA: Tramadol; IBU: Ibuprofen; DIC: Diclofenac)
Validation of the method

The identification of the analytes was based on their retention times (paracetamol, 7.0 min; salicylic acid, 8.2 min; metamizol, 13.5 min; tramadol, 15.7 min; ibuprofen, 16.8 min; and diclofenac, 18.2 min) and absorption spectra. The working wavelengths from the maximum absorbance for each drug were: 244 nm for paracetamol and metamizol; 225 nm for salicylic acid and ibuprofen; and 275 nm for tramadol and diclofenac. The specificity and sensitivity of method were improved since the analytes were identified and quantified at the wavelength of the maximum response, and they can be discriminated from their metabolites and other plasma components coextracted and coeluted at near retention times.

The linearity of method was studied in a concentration range 0.1-20.0 µg/mL, by performing five replicates of each concentration level. All solutions were extracted using SPE, and analyzed by HPLC-PDA. The limit of detection (LOD) was determined from the lowest concentration with a signal to noise ratio of at least three, and the limit of quantification (LOQ) as ten times the signal to noise ratio in blank plasma. Table 1 presents the results of linearity and limits of detection. LODs of 80 ng/mL for paracetamol, 40 ng/mL for diclofenac and 10 ng/mL for aspirin [18] and 0.8 ng/mL for tramadol [19] in human plasma were obtained when LC-MS was used. LODs of 0.3 ng/mL for ibuprofen and diclofenac in water were obtained using LC-MS [20]. When HPLC-UV was applied, other authors reported LODs higher than those obtained in this work, for salicylic acid or paracetamol in serum [9], plasma [13] or tablets [21]. Cueva-Mestanza et al. [22] presented values of 4 ng/g for ibuprofen in sediments.

The within-day and between-day precision was calculated at three concentration levels (0.5; 5 and 20 µg/mL) of the six analgesics studied, obtaining good coefficients of variation, according to international guidelines [15, 16]: 0.5-6.4% for within-day study, and 2.0-7.9% for between-day study, in five replicates (Table 2). Using the same extraction procedure (SPE), Pailler et al. [20] reported mean coefficients of variation of 13% and 8.5% for ibuprofen and diclofenac, respectively. Ohwaki et al. [13] obtained values of 4.9% and 6.1% for salicylic acid in within-day and between-day tests, respectively. Suenami et al. [18] published coefficients of variation for paracetamol and aspirin higher than those obtained in this work, but lower for diclofenac and ibuprofen.

Applications

The method validated was applied to 16 plasma samples from analgesic users. Table 3 shows the results, and as can be seen, paracetamol was the most frequently detected, followed by ibuprofen, tramadol and diclofenac. Salicylic acid and metamizol were only detected in one case. The concentrations found in real plasma samples correspond to toxic/lethal levels for paracetamol in 4 cases, metamizol in 1 case, tramadol in 4 cases and diclofenac in 1 case.
case [23]. The mean levels were 36.1 µg/mL for paracetamol, 37.8 µg/mL for salicylic acid, 27.7 µg/mL for metamizol, 14.5 µg/mL for tramadol, 2.0 µg/mL for ibuprofen and 25.0 µg/mL for diclofenac. Figure 3 shows the chromatogram for a real case. These results are in agreement with several studies reported, with a mean level of 38.3 µg/mL for paracetamol in 148 intoxications [24] and a concentration of 8.6 µg/mL for metamizol in a suicide by multidrug ingestion [25]. Between all cases studied, there was four deaths associated to tramadol consumption, presenting plasma levels comparable to those published by other authors: 7.7-48.3 µg/mL [26], 8 µg/mL [27] and 1.1-12.0 µg/mL [28].

Table 3. Concentrations of analgesics in 16 real plasma samples

<table>
<thead>
<tr>
<th>Analgesic</th>
<th>Frequency</th>
<th>Concentration range in real cases (µg/mL)</th>
<th>Therapeutic concentration (µg/mL)</th>
<th>Toxic/lethal concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol</td>
<td>9/16</td>
<td>1.8-124.7</td>
<td>10-20</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>1/16</td>
<td>37.8</td>
<td>20-100</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Metamizol</td>
<td>1/16</td>
<td>27.7</td>
<td>1.6-11.0</td>
<td>106 (1 case)</td>
</tr>
<tr>
<td>Tramadol</td>
<td>4/16</td>
<td>2.2-32.8</td>
<td>0.1-0.8</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>5/16</td>
<td>0.5-4.6</td>
<td>20-30</td>
<td>400-811 (3 cases)</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>2/16</td>
<td>1.3-48.7</td>
<td>0.1-2.2</td>
<td>60 (1 case)</td>
</tr>
</tbody>
</table>

*Moftat et al, 2004 [23]*

Conclusions

The developed analytical method, simple and inexpensive, enables the simultaneous determination of paracetamol, salicylic acid, metamizol, tramadol, ibuprofen and diclofenac in human plasma. HPLC-PDA is an alternative technique when mass spectrometry is not available because the validation parameters are satisfactory according to the concentration levels found in plasma samples from intoxicated subjects. Moreover, the chromatographic responses can be maximized, working at the wavelength of maximum absorption for these compounds. Solid-phase extraction was proved to be a suitable procedure for analyzing plasma samples, monitoring a single dose and demonstrating exposure to analgesics in forensic cases.

Acknowledgements

This research was supported by the project 10PXIB208089PR, from Xunta de Galicia.

References

17. Rodríguez T, Quintana JB, Carpintero J, Carro AM, Lorenzo RA, Cela R (2003) determination of acidic drugs in sewage water by
Simultaneous determination of six analgesics in human plasma using solid-phase extraction and liquid chromatography


