An Enzymic Digestion and Solid-Phase Extraction Procedure for the Screening for Acidic, Neutral, and Basic Drugs in Liver Using Gas Chromatography for Analysis

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Abstract

Analysis of liver specimens is an important issue in forensic toxicology, but suitable workup and extraction methods for general screening purposes have been lacking until now. A workup and extraction scheme based on a recently developed procedure for the screening of biological fluids was developed that can be used for the screening of acidic, neutral, and basic drugs in liver. This method uses a single solid-phase extraction (SPE) column and gas chromatography–flame ionization detection (GC–FID) for the final analysis. First, the homogenized liver sample is sonicated and centrifuged; the resulting supernatant is applied to the SPE column. Elution of acidic, neutral, and some weakly basic drugs is then performed with acetonitrile–chloroform and analyzed by GC–FID. Next, the pellet of tissue material obtained from the centrifugation is enzymically digested by subtilisin Carlsberg. This frees the drugs bound to the liver tissue. The resulting clear liquid is brought to the reconditioned SPE column. A wash step is introduced to remove acidic and neutral interferences and the basic drugs can then be eluted with ammoniated ethyl acetate. Using 100-mg wet liver samples spiked with 2 µg of amounts of various drugs, recoveries were 70–102% with relative standard deviations less than 9%. The resulting GC–FID chromatograms were virtually free of endogenous interferences. With the SPE columns currently used, endogenous interferences and the basic drugs can then be eluted with ammoniated ethyl acetate. Using 100-mg wet liver samples spiked with 2 µg of amounts of various drugs, recoveries were 70–102% with relative standard deviations less than 9%. The resulting GC–FID chromatograms were virtually free of endogenous interferences. GC–nitrogen-phosphorous detection detected smaller amounts of nitrogen-containing drugs, again without endogenous interferences. With the SPE columns currently used, which contain a bed mass of 130 mg, the liver samples should be smaller than 200 mg because the endogenous compounds obtained after the digestion of the tissue will overload the column, which results in a lower recovery of the drugs of interest. Drugs that decompose under the digestion conditions (pH 10.5 at 60°C for 1 h) may be lost in the present procedure. This phenomenon is being investigated further.

Introduction

In forensic toxicology, blood and urine may be available only in small amounts or not at all. Moreover, interpretation may be difficult because blood levels are frequently site-dependent, with heart blood giving higher levels than femoral blood (1). Therefore, analysis of tissue material is of interest. Before carrying out the analysis, the relevant drugs must be extracted from tissue material. Extraction techniques must be rapid, efficient, and give good recoveries for most of the relevant drugs. In addition, they must provide clean extracts. Meeting these requirements has proven to be a very difficult task for forensic scientists.

Until now, the commonly used methods for extracting drugs from tissue samples were direct solvent extraction or liquid–liquid extraction after pretreatment of the tissue with a protein precipitant or acid hydrolysis of the tissue. However, direct extraction suffered from solvent emulsion and resulted in dirty extracts; protein precipitation gave low yields of drugs; and acid hydrolysis was not suitable for some acid or heat labile drugs (2). In 1977, Osselton et al. (3–5) first reported on enzyme digestion for releasing some acidic, neutral, and basic drugs from liver tissue. Compared with other extraction methods, the enzymic digestion yielded far higher recoveries, which indicated that enzymic digestion may be the best pretreatment method for general screening. Since then, some other enzymic digestion methods have been published (6–8). Generally, the enzymic digestion is followed by liquid–liquid extraction.

Over the past decade, solid-phase extraction (SPE) has emerged as a powerful tool for the extraction of biological fluids for toxicological drug analysis (9–11). SPE of tissue samples has been reported as well (12–14). However, these methods are less suitable for drug screening because they were established for a single drug or a group of relevant drugs. The only SPE method for drug screening of tissue has been reported by Cordonnier et al. (15).

Recently, a single column SPE procedure was developed in our laboratory for the screening for acidic, neutral, and basic drugs in plasma, whole blood, and urine. It has been demonstrated to be useful for drug screening (16–20). In this study, we investigated whether the screening procedure for biological fluids could be adapted for drug screening in liver tissue.
Materials and Methods

Materials

All reagents were analytical-grade (Merck, Darmstadt, Germany), with the exceptions of ethyl acetate, acetone (Lab-Scan, Dublin, Ireland), and chloroform (Westburg, Leusden, The Netherlands), which were HPLC-grade. The test drugs were from commercial suppliers and were of pharmaceutical quality. Stock solutions of the individual drugs (1 mg/mL) were prepared by dissolving the appropriate amount of drug in methanol–ethyl acetate (1:1, v/v). For spiking the samples, the stock solutions were diluted 10 times with methanol–ethyl acetate. The chromatographic standard solution was prepared by diluting the stock solution of prazepam with ethyl acetate to 20 μg/mL.

Subtilisin Carlsberg (11 units/mg) was purchased from Sigma Chemical (St. Louis, MO), and papain (0.57 units/mg) was purchased from Fluka (Buchs, Switzerland). Bond-Elut Certify columns with a capacity of 10 mL (130 mg sorbent) and the Vac Elut vacuum manifold were supplied by Varian Sample Preparation Products (Harbor City, CA). Phosphate buffer (0.1 mol/L, pH 6.0) was prepared by dissolving 6.81 g potassium dihydrogen phosphate in 450 mL deionized water, adjusting the pH with 1 mol/L potassium hydroxide, and making the total volume up to 500 mL with deionized water. Acetic acid (1 mol/mL, pH 2.4) was prepared by diluting 5.75 mL glacial acetic acid to 100 mL with deionized water. Acetate buffer (1 mol/mL, pH 4.0) was prepared by mixing 0.575 mL glacial acetic acid with 1.6 mL 1 mol/L potassium hydroxide and 80 mL deionized water. If necessary, the pH was adjusted to 4.0, and the volume was made up to 100 mL with deionized water. Tris-base solution 1 mol/L was prepared by dissolving 12.1 g tris(hydroxymethyl)aminomethane in 100 mL deionized water.

Subtilisin tris-base solution was prepared by dissolving 2 mg subtilisin Carlsberg in 4 mL 1 mol/L tris-base solution before use. Papain solution was prepared by dissolving 250 mg papain in 3 mL phosphate buffer (pH 7.4) and then adding 0.25 mL cysteine (0.1 mol/L) and 0.1 mL EDTA (0.1 mol/L). Ammoniated ethyl acetate (2%) was prepared daily by adding 1 mL ammonia (25%) to 49 mL ethyl acetate in an Erlenmeyer flask with a glass stopper and sonicating for 10 min.

Figure 1. GC-NPD chromatograms of Fraction A and Fraction B after SPE of liver tissue digested by subtilisin and using the same procedure as Chen et al. (17). Peak 1 is from prazepam (chromatographic standard).

Figure 2. Outline of the SPE procedure for screening liver samples using GC for analysis.
5 min. Acetone–chloroform was prepared by mixing equal volumes of each. Phosphoric acid (10%) was prepared by mixing 7.35 mL phosphoric acid (85 wt%) with 92.65 mL deionized water.

**Instrumentation**

A Sonicor sonic bath was purchased from Sonicor Instrument Corporation (Farmingdale, NY). A Megafuge 1.0 centrifuge, which was purchased from Heraeus Sepatech (Osterode/Harz, Germany), was used at 6000 × g (4000 rpm). An Ultra Turrax homogenizer was purchased from Wilten (Utrecht, The Netherlands). Analyses were performed with a HP-5890 series II GC (Hewlett-Packard, Avondale, PA) equipped with a HP-7673 automatic sampler, a flame-ionization detector (FID), and a nitrogen-phosphorous detector (NPD). The column was a 30-m HP-1 fused-silica capillary column with a 0.53-mm internal diameter and a film thickness of 0.88 μm. The oven temperature program for FID was 2 min at 80°C, increased 20°C/min to 215°C, increased 5°C/min to 285°C, and held 5 min at the final temperature. For NPD, the program was 2 min at 150°C, increased 20°C/min to 215°C, increased 5°C/min to 285°C, and held 5 min at the final temperature. The injector and detector temperatures were 275 and 310°C, respectively.

**Spiking liver tissue**

Calf liver (1 g) was finely minced and mixed with 3 mL water in an Ultra Turrax homogenizer. Of the homogenized liver, 0.4 mL (equivalent to 100 mg of wet liver) was spiked with 2 μg of each test drug (20 μL of 0.1 mg/mL test drug solution). The spiked samples were vortex mixed for 30 s and equilibrated on a waterbath at 37°C for 1 h. Livers were also spiked at 1 mg and 0.1 μg/100 mg (see Figure 1).

**Sample pretreatment**

One milliliter of deionized water was added to 0.4 mL of spiked liver homogenate. The mixture was sonicated for 5 min and then centrifuged at 6000 × g for 10 min. The supernatant was applied to the SPE column at step 2 of the procedure below.

Subtilisin tris-base solution (1 mL) was added to the pellet. The mixture was vortex mixed for 10 s and digested at 60°C for 1 h. After cooling down to room temperature, the pH was adjusted to 6–7 with 10% H₃PO₄. The suspension was centrifuged at 6000 × g for 10 min. The supernatant was applied to the SPE column at step 7 of the procedure below.

**SPE procedure for screening liver tissue**

The extraction procedure is outlined in Figure 2. The procedure consisted of 13 steps: 1. The column was preconditioned with 2 mL of methanol and then with 2 mL of phosphate buffer (pH 6.0). 2. The supernatant was applied onto the column after sonication and passed through completely at a flow rate of 1.5 mL/min. 3. The column was washed sequentially with 1 mL deionized water and 0.5 mL acetate buffer (pH 4.0). 4. The column was dried under full vacuum (15 in. Hg) for 4 min. After rinsing the column with 50 μL methanol, it was dried again for 1 min. 5. A labeled evaporation tube was placed into the column manifold after rinsing the column outlet and manifold with water. Then 4 mL of acetone–chloroform (1:1) was added to the column reservoir and eluted completely at a...
flow rate of 0.8 mL/min. This eluate is Fraction A. 6. The column was reconditioned with 2 mL of phosphate buffer (pH 6.0). 7. After digestion, the supernatant was applied to the column and passed through at a flow rate of 1–1.5 mL/min. 8. The column was washed sequentially with 1 mL of deionized water, 1 mL 1 mol/L HAc (pH 2.4), and 2 mL acetone–chloroform (1:1). 9. The column was dried as in step 4. 10. After wiping the column outlet and manifold basin with tissue, another labeled evaporation tube was placed into the manifold basin. Ammoniated ethyl acetate (2%, 2 mL) was added to the column reservoir; the flow rate was 0.5 mL/min. This eluate is Fraction B. 11. To each tube, 100 µL of chromatographic standard solution (prazepam, 20 µg/mL) was added. The eluate was then evaporated in a water bath at 37°C under a nitrogen stream until about 100 µL of solvent remained in each tube. 12. One microliter of the remaining solvent was injected onto the GC. The ratio of the peak heights of the analyte to that of prazepam was used for quantitation. 13. Absolute extraction yields were determined by comparing the response ratios of the extract with the calibration graph from the pure substance in the concentration range of 4–24 µg/mL with a constant concentration of prazepam (20 µg/mL). For some weakly basic drugs that appeared in both Fractions, the extraction yield was calculated by summing up the percentage in both Fractions.

Results and Discussion

Homogenized liver tissue cannot be applied directly onto the SPE columns because this will result in the clogging of the column. Different pretreatment procedures of liver samples were carried out. Criteria for judging quality of the extracts were cleanliness of the extracts and the extraction yields of the drugs in the spiked samples. It must be emphasized that high extraction yields of the drugs in the spiked samples does not automatically mean that these yields will also be obtained in authentic specimens; however, high extraction yields in spiked sample are at least a prerequisite. For the calculation of absolute extraction yields, a chromatographic standard (prazepam) was added at the end of extraction procedure, just prior to the injection step in order to correct for difference in the final volume of the extracts and for difference in the chromatographic process.

Sonication and centrifugation

Sonication followed by centrifugation as the only pretreatment of the homogenized liver sample gave low recoveries, especially for basic drugs. Apparently, some basic drugs remain bound to the particulate cell material and are subsequently spun down in the pellet. However, acidic drugs such as barbiturates showed good recoveries, usually between 80 and 100%.

Enzymic digestion

Different types of enzymes were used to digest the liver tissue before sample application. Shankar et al. (7,8) found that papain gave the best result. However, digestion with papain resulted in very dirty extracts, which caused substantial interferences in the subsequent GC analyses. Shankar et al. (7,8) used high-performance liquid chromatography (HPLC) with ultra-violet (UV) detection at 254 nm for basic drugs and 240 nm for barbiturates. The impurities detected by GC may not be detected under these conditions. In agreement with the literature (3–5), the protease subtilisin Carlsberg at a pH of 10.5 and a temperature...
of 55–60°C digested the tissue very efficiently. It must be noted that during digestion the pH drops to 8–9. However, when applying the digested material to the SPE procedure used for blood and urine (17), the first fraction, Fraction A, which contained the acidic and neutral drugs, gave chromatograms with many interfering peaks when analyzed with GC-FID (Figure 3A). These interferences appear to be a result of lipophilic compounds such as fatty acids. When using GC–NPD, cleaner chromatograms were obtained (Figure 1A), but they were still not considered to be useful analyses. In contrast, Fraction B was much cleaner and contained only a small number of impurity peaks (Figures 1B and 3B). Because the lipoid interferences are of acidic to neutral character, they were washed away by the acetone–chloroform eluent used to obtain Fraction A. The interferences seen in Fraction B may then be due to compounds with a basic character. It must also be noted that GC–MS analysis of Fraction A is very complicated, and moreover, the many interferences present may easily contaminate the instrument.

Combination of sonication/centrifugation and enzymic digestion with subtilisin Carlsberg

The two tissue pretreatment procedures outlined previously were combined, which resulted in an extraction procedure as described in the Method section and schematically presented in Figure 2. Typical chromatograms of spiked samples that were analysed by GC–FID are depicted in Figure 4. In order to get optimal results, the following criteria were investigated in more detail.

The pH of the enzymic digestion

According to the manufacturer, Novo, the optimal pH for subtilisin digestion is 9–11 (22). Other authors stated that lower pH values can be used (6–8). At pH 10.5, one can expect that some drugs, such as esters like atropine, may decompose. Therefore, pH values between 7.5 and 10.5 were investigated. However, at pH values less than 10.5, the digestion did not result in clear solutions. The remaining particulate material clogged the columns, and centrifugation to remove the particulate material gave low recoveries of some drugs in the supernatants. Thus, a digestion pH of 10.5 appeared to be necessary.

Influence of the type of buffer in the enzymic digestion step

The use of a tris buffer pH 10.5, which is usually recommended for subtilisin digestion, resulted in a few impurities in Fraction B (Figure 4B, retention time, 5–7 min). Replacing the tris buffer with a 1 mol/L K$_2$HPO$_4$ solution with the pH adjusted to 10.5 with 1 mol/L KOH, resulted in a much cleaner chromatogram for Fraction B (Figure 5) without affecting the efficiency of the digestion itself.

Influence of the amount of liver tissue

Extractions were carried out according to the procedure outlined in Figure 2 with liver portions of 100–500 mg and a fixed analyte concentration. Figure 6 shows that with 100 mg liver tissue, recoveries of approximately 70–100% were found for all drugs investigated. With higher amounts of tissue the extraction yields dropped substantially, to as low as 20%, except for mepivacaine, which remained rather constant (between 70–80%). Moreover, with higher amounts of liver matrix, more interfering peaks were seen in the chromatograms. Another phenomenon was observed: ketazolam usually eluted in Fraction B when 100 mg liver was applied, but it appeared in Fraction A when 200 mg of tissue or more was used. This indicates

### Table I. Postmortem Concentrations of Drugs in Liver

<table>
<thead>
<tr>
<th>Drug</th>
<th>Postmortem liver concentrations (µg/g)</th>
<th>Number of fatalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codeine</td>
<td>0.1–45</td>
<td>5</td>
</tr>
<tr>
<td>Diazepam</td>
<td>11–13</td>
<td>12</td>
</tr>
<tr>
<td>Doxepin</td>
<td>22–38</td>
<td>32</td>
</tr>
<tr>
<td>Clutethimide</td>
<td>63–141</td>
<td>89</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>20–96</td>
<td>62</td>
</tr>
<tr>
<td>Mepivacaine</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Meprobamate</td>
<td>58–360</td>
<td>148</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>0.4–175</td>
<td>44</td>
</tr>
<tr>
<td>Methadone</td>
<td>0.05–50</td>
<td>6</td>
</tr>
<tr>
<td>Methaqualone</td>
<td>26–89</td>
<td>55</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>8–220</td>
<td>90</td>
</tr>
<tr>
<td>Pentobarbital</td>
<td>23–550</td>
<td>130</td>
</tr>
<tr>
<td>Promethazine</td>
<td>23–31</td>
<td>27</td>
</tr>
<tr>
<td>Secobarbital</td>
<td>25–605</td>
<td>271</td>
</tr>
<tr>
<td>Trimipramine</td>
<td>224–544</td>
<td>384</td>
</tr>
</tbody>
</table>

* Data from Moffat (21). These concentrations are associated with suspected drug overdose.
that higher amounts of tissue material on the SPE column cover the binding sites of the functional groups in the Bond-Elut Certify sorbent, thus preventing the latter from adequately retaining the drugs of interest. This hypothesis would also explain the constant recovery of mepivacaine in Figure 6 if one assumes that the affinity of mepivacaine to the binding sites of the SPE column is larger than that of the matrix components. Therefore, only 100 mg of liver tissue was used in subsequent experiments. In general, with this amount of liver, detection of 1 μg of drug in 100 mg of wet tissue is possible with GC-FID, which should be quite sufficient in forensic toxicology because drug concentrations in liver often exceed 10 μg/g in fatal cases (Table 1). If desired, lower concentrations can be detected with GC-NPD or GC-MS.

Recoveries with the optimized sonication–centrifugation–digestion method

Utilizing the procedure of Figure 2 and optimized as described above, recoveries for 20 drugs were determined using GC-FID for the final analysis at a concentration level of 20 μg/g liver (i.e., 100 mg liver with 2 μg drug). The results are presented in Table II. With the exception of benzocaine and promethazine, extraction yields of 70–100% were obtained. The precision expressed as relative standard deviation (RSD) was below 8.4%. Care should be taken for some weakly basic drugs (e.g., benzodiazepines and methaqualone) with pKₐ values around 3 that appear in both Fractions. Part of these drugs may be washed away by acetone–chloroform before eluting Fraction B. Benzocaine is a weakly basic substance (pKₐ 2.8). Moreover, benzocaine has an ester functional group. We would expect benzocaine to appear in both Fractions (Fractions A and B). The absence of benzocaine in Fraction B may be a result of decomposition after enzymic digestion or of being washed away during the washing step with acetone–chloroform prior to the elution of Fraction B.

Promethazine is a phenothiazine drug that may decompose under alkaline conditions, especially under the influence of light. However, the recoveries still seem to be sufficient to detect the presence of these types of drugs.

Conclusion

The presented work-up and extraction procedure for the systematic analysis of liver tissue fulfilled the two major criteria of providing sufficiently high recoveries of a wide selection of toxicologically relevant drugs and giving GC-FID chromatograms with minimal interference from endogenous tissue compounds. This is substantiated by the following observations: 1. The binding affinity of acidic and neutral drugs to liver tissue is relatively weak. Consequently, these drugs can be liberated and extracted by SPE after sonication centrifugation of the liver homogenate. Coextraction of potentially interfering endogenous compounds is minimal under these conditions. 2. The binding affinity of basic drugs to liver tissue is much stronger, which makes the digestion of the tissue material necessary to liberate these drugs and available for SPE. Although digestion also results in the freeing of a great many endogenous compounds, the acidic or neutral character of these compounds makes it possible to remove them by applying an acidic wash to the SPE column. The basic drugs can then be eluted in the next step by using ammoniated ethyl acetate, and this basic fraction is virtually free of endogenous interferences. 3. The combination of sonication and enzymic digestion was found to be an effective technique for pretreatment of liver. The optimal pH of subtilisin digestion is 10.5 at a temperature of 55–60°C.

A phosphate buffer gave cleaner chromatograms for Fraction B than the tris buffer. BondElut Certify columns can be used for the extraction of a wide range of drugs in liver. For columns with 130 mg sorbent, the amount of liver should be smaller than 200 mg in order to obtain acceptable and reproducible recoveries.

It should be noted that the present work was carried out with spiked liver samples that were left to equilibrate for at least 1 h at 37°C prior to preparation. Further work on liver samples from case work is in progress. We are also investigating the fate of ester drugs that may decompose during the enzymic digestion.

Acknowledgment

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References


22. Novo Industry AIS, Enzymes Division, Application Note, (1979)