Dose Determination of Activated Charcoal in Management of Amitriptyline-Induced Poisoning by Reversed-Phase High-Performance Liquid Chromatography

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Abstract

Purpose: To assess the doses of activated charcoal currently used in the management of acute amitriptyline-induced drug poisoning and explore the possibility of using lower doses.

Methods: Albino male Wistar rats, weighing 200 ± 20 g, were used for the study. The animals were divided into four groups of eight animals each. The concentration of amitriptyline in rat plasma was measured by high performance liquid chromatography (HPLC) for dose determination of activated charcoal. Chromatograms were established with acetonitrile: 70 mM KH₂PO₄ buffer (60: 40, v/v) solvent system on an Xterra® ms C18 SUM column (5 µm, 3.9 x 150 mm) and pH was adjusted to 4.5 with ortho-phosphoric acid. Mobile phase flow rate was 1 ml/min and ultraviolet (UV) detection was at 293 nm. Validation of the method was performed to determine its selectivity, linearity, precision, as well as limits of detection (LOD) and of quantification (LOQ).

Results: Standard curves were linear, r² = 0.996, for amitriptyline over the concentration range 10 - 60 ng/ml. Recovery (98.3 to 100.85 %) was in the selected concentration range of 10 - 60 ng/ml. The LOD and LOQ of the method for amitriptyline were 0.109 and 0.332 µg/ml, respectively. The validated method was successfully applied to measure plasma concentrations of amitriptyline and to measure the doses of activated charcoal currently used in the management of acute amitriptyline drug poisoning.

Conclusion: The proposed RP-HPLC method enables determination of amitriptyline with good separation and resolution of the chromatographic peaks. Validation revealed that the method is sensitive, accurate and selective. Using half of the standard dose of the activated charcoal gave a comparable effect to the standard dose in reducing drug concentration in the blood. While, using quarter of the standard dose of activated charcoal does not have a cleared effect.

Keywords: Amitriptyline, Activated charcoal, Drug poisoning, Reversed-phase high-performance liquid chromatography

INTRODUCTION

Poisoning is a global public health concern. According to WHO data, in 2004, an estimated 346,000 people died worldwide from unintentional poisoning. Of these deaths, 91 % occurred in low- and middle-income countries [1]. In Saudi Arabia, annual nationwide data on poisoning patterns are scarce and incomplete. Most previous studies conducted in Saudi Arabia
that focused on poisoning were city- or region-specific and failed to present the overall epidemiological characteristics and long-term trends in poisoning [2-7].

Activated charcoal has been used for the last century for gastric decontamination [8]. The amount of drug that adsorbs to the activated charcoal is dependent on the charcoal-to-drug ratio, with the optimal ratio proposed to be 10:1 [9]. As the dose of drug is rarely known, a standard dose of charcoal is normally given. The position statement for a single dose of activated charcoal suggests a dose of 10 to 25 g (or 0.5 - 1.0 g/kg) of body weight in a child up to 1 year of age, 25 to 50 g (or 0.5 - 1.0 g/kg) in children 1 to 12 years of age, and 25 to 100 g in adolescents and adults. However, most children up to 12 years of age receive a dose of 1 g/kg of body weight [10], although there is no single correct dose of activated charcoal.

Thus, there is a need to design a study to test the use of lower doses than the current standard dose of activated charcoal used in the treatment of acute drug poisoning, especially for drugs used in small doses (such as tricyclic antidepressants) to determine the appropriate dose of activated charcoal. It is evident from the literature that tricyclic antidepressants are identified as one of the most frequently ingested substances in self-poisoning along with paracetamol, benzodiazepines and alcohol [11]. There is also evidence that the number of deaths relative to the number of prescriptions issued is significantly higher for tricyclics than for other antidepressants [12].

The objective of this study was to assess the activated charcoal doses currently used in the management of acute drug poisoning and hence explore the possibility of using lower doses of activated charcoal, especially for drugs used in small doses.

**EXPERIMENTAL**

**Materials**

Amitriptyline and clomipramine (IS) from Sigma-Aldrich (St Louis, MO, USA) were kindly provided by toxicology unit, College of Medicine, King Saud University, Riyadh, Saudi Arabia. HPLC-grade acetonitrile was obtained from Panreac Chemicals (Barcelona, Spain), potassium dihydrogen phosphate and activated charcoal were procured from Winlab Ltd. (Maidenhead, Berkshire, UK) and water was produced in the laboratory by a Milli-Q purification system (Millipore Corp., Billerica, MA, USA). All other reagents used were at least of analytical grade.

**Method development**

**Instrumentation and chromatographic conditions**

High performance liquid chromatography (HPLC, Waters model 2695 Alliance separation module), equipped with UV detector. The chromatographic identification was carried out at room temperature (25 ± 1 ºC). The mobile phase consisted of acetonitrile and potassium dihydrogen phosphate buffer (70 mM ), in the ratio of 60:40 v/v, which was delivered isocratically at a flow rate of 1 ml/min. Xtera® ms C18 SUM (5 µm, 3.9 × 150 mm) column was utilized to elute the compounds of interest at a $\lambda_{\text{max}} = 293$ nm for the analysis of amitriptyline.

**Selection of mobile phase**

Different combinations of solvent systems of acetonitrile, buffer (KH$_2$PO$_4$): acetonitrile and formic acid: water and methanol: water: acetonitrile etc were tried in order to determine the best conditions for the separation and optimization of amitriptyline. The mobile phase consisting of acetonitrile and 70 Mm potassium dihydrogen phosphate buffer (KH$_2$PO$_4$) with pH adjusted to 4.5 in the ratio of (60:40 % v/v) was selected as it gave high resolution for amitriptyline with minimal tailing.

**Preparation of standard and calibration samples**

Stock standard solutions of 1 mg/ml amitriptyline and the internal standard (IS) clomipramine were individually prepared in acetonitrile. These stock solutions were kept frozen and used within 2 weeks. The working standard solution for each compound was prepared by serial dilution of the stock solution with acetonitrile. Calibration standards of plasma samples for UV detection were prepared by spiking the plasma with working solutions of amitriptyline to make the final concentrations of 10, 15, 20, 25, 30, 35, 40, 45, 50 and 60 ng/ml.

**Sample preparation**

Plasma samples kept at -80 °C were thawed for one hour and then vortexed for 30 s at room temperature before extraction to ensure homogeneity. Amitriptyline was extracted from the plasma with acetonitrile as the precipitation solvent. To 200 µl of plasma containing
amitriptyline, IS (110 ng/ml) was added in a 1.5-
ml capped centrifuge tube with vortex mixing. 
Acetonitrile (140 μl) and 120 μl of concentrated 
potassium phosphate solution (1 g/ml) were 
added. The mixture was shaken and centrifuged 
at 2500 × g for 15 min. The supernatant was 
transferred to another Eppendorf tube and 20 μl 
of this was injected onto the column for the 
analysis of amitriptyline by RP-HPLC.

Method validation

Method validation was carried out according to 
ICH guidelines [13] in rat plasma in order to 
evaluate the method for linearity of response, 
accuracy, precision, recovery, limit of detection and 
quantification, selectivity, stability of analyte 
during processing, and storage.

Linearity
The linearity of an analytical method is its ability 
to elicit test results that are directly proportional 
to the concentration of analyte in samples within 
a given range. Thus, linearity refers to the 
relationship of concentration and assay 
measurement [13]. The linearity of the proposed 
method was evaluated by using calibration 
curves to calculate coefficient of correlation and 
intercept values.

Precision, accuracy and recovery
For precision and accuracy (n = 6) of the method 
was determining by using calibration standard 
solution (30 ng/ml) of amitriptyline and results 
was expressed in terms of % RSD. Recovery 
studies were carried out by applying the method 
to drug samples to which known amount of 
amitriptyline corresponding to higher 
concentration 60 ng/ml and lower concentration 
10 ng/ml. At each level, six determinations were 
performed.

Limit of detection and of quantification (LOD and LOQ)
For determination of LOD and LOQ, blank 
plasma samples (without AMT and IS) were 
injected in triplicate for peak area calculations. 
LOD and LOQ were determined from the slope 
(S) of the calibration curve and SD of the 
response for the blank samples as follows: LOD 
= 3.3 × SD/S; and LOQ = 10 × SD/S.

Selectivity and specificity
The selectivity of the method was checked by 
injecting solution of amitriptyline. It was observed 
that sharp peaks for IS and amitriptyline occurred 
at retention times of 4.5 and 6.15 min, 
respectively. These peaks were not present in 
the chromatogram of blank plasma. The 
specificity of the method was assessed by 
comparing chromatograms obtained from drug 
standards with that obtained from plasma of 
animals. The retention times of the drug 
standards and the drugs from treated animals 
were found to be same, indicating the method 
was specific and selective because of no 
interference was found in plasma (Fig 1A - D).

Stability
The stability (short term) of standard and 
amitriptyline sample solutions was carried out by 
analyzing the drug after 24 and 48 h at room 
temperature and - 20 °C against fresh standard 
solutions. Long-term stability was evaluated after 
storing samples at -80 °C for 30 days. All 
stability experiments were carried out against 
freshly spiked calibration standards.

Method application

Animals
Albino male Wistar rats weighing approximately 
200 ± 20 g were obtained from the Experimental 
Animal Care Center, College of Pharmacy, King 
Saud University, Riyadh, Saudi Arabia. The 
animals were kept under controlled 
environmental conditions (25 ºC and a 12 h 
light/dark cycle). Animals were given free access 
to pulverized standard rat pellet food and tap 
water. The protocol of this study has been 
approved (clearance number 021015-0618; 
March 10, 2013) by Research Ethics Committee 
of College of Pharmacy, King Saud University, 
Riyadh, Saudi Arabia, and the animals were 
handled in accordance with the Guide for the 
Care and Use of Laboratory Animals by National 
Institute of Health[14].

Experimental design
The animals were divided into four groups of 
eight animals each. Group I served as positive 
control received only 1 ml of amitriptyline (2.5 
mg/ml) by oral route. Group- II was administered 
with 1 ml of amitriptyline (2.5 mg/ml) and af-

1 ml of activated charcoal (200 mg/ml dose, i.e., the standard dose of the activated charcoal). Standard dose of the activated charcoal have been measured on the basis of the activated charcoal dose, 1 g/kg of the body weight. After two hours of dosing the animals were anesthesized and blood was collected from heart. Plasma was separated by centrifugation at 2500 x g for 10 min and was transferred to pre-labeled eppendorf tubes for subsequent analysis of amitriptyline concentration using the developed method.

**Statistical analysis**

Data were statistically analyzed using the statistics program (Sigma plot V12) and are given as mean ± SEM. Differences between
groups were determined by one-way analysis of variance (ANOVA) using Student-Newman-Keuls method for all pair-wise multiple comparison procedure. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

The method was developed and validated according to ICH guidelines. The linear regression analysis indicated a good linearity over a wide concentration range (10 – 60 ng/ml) with regard to peak area. The mobile phase compositions were prepared with appropriate ratios of acetonitrile, water and buffer (70 Mm). The adjustment of pH 4.5 was made using orthophosphoric acid. It was detected that the best determination of amitriptyline was attained with a mobile phase composed of acetonitrile and buffer (pH 4.5) at (60:40 % v/v) ratio and a flow rate of 1 ml/min.

**Calibration curve and linearity**

The test was test on ten concentrations (10, 15, 20, 25, 30, 35, 40, 45, 50, 60 ng/ml) and was repeated three times for each concentration. No significant difference was observed in the slopes of standard curves. The observed straight line equation was, \( y = 0.033x + 0.0056 \) with \( r^2 \) value of > 0.9996.

**Precision, accuracy and recovery**

The precision of the developed analytical method was done by measuring the standard amitriptyline concentration (30 ng/ml). Measurement was done repeatedly six times for each injection. The relative standard deviation (RSD) of the results was found to be 0.51, while the percentage recovery was found to be 100.056 ± 0.51 %, as shown in (Table 1).

<table>
<thead>
<tr>
<th>S/no.</th>
<th>Measured concentration (ng/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30.07</td>
<td>100.23</td>
</tr>
<tr>
<td>2</td>
<td>30.12</td>
<td>100.40</td>
</tr>
<tr>
<td>3</td>
<td>29.87</td>
<td>99.57</td>
</tr>
<tr>
<td>4</td>
<td>30.23</td>
<td>100.80</td>
</tr>
<tr>
<td>5</td>
<td>29.98</td>
<td>99.93</td>
</tr>
<tr>
<td>6</td>
<td>29.83</td>
<td>99.43</td>
</tr>
<tr>
<td>Mean</td>
<td>30.017</td>
<td>100.05</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.153</td>
<td>0.51</td>
</tr>
</tbody>
</table>

The accuracy of the assay method was evaluated at two concentration levels of amitriptyline, i.e., higher concentration (60 ng/ml) and lower concentration (10 ng/ml) and repeated six times for each injection. The percentage of recoveries and % RSD were calculated from the slope and \( y \)-intercept of the calibration curve. The recovery values obtained were in the range of 98.3 to 100.85 % confirming accuracy of the developed method (Table 2). The percentage recovery of the lowest concentration was 99.71 ± 0.99 % and that of the highest concentration was 100.001 ± 0.185 %.

<table>
<thead>
<tr>
<th>S/no.</th>
<th>Lowest concentration (10 ng/ml)</th>
<th>Recovery (%)</th>
<th>Highest concentration (60 ng/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10.33</td>
<td>99.15</td>
<td>60.1</td>
<td>99.7</td>
</tr>
<tr>
<td>2.</td>
<td>10.31</td>
<td>98.3</td>
<td>60.32</td>
<td>99.87</td>
</tr>
<tr>
<td>3.</td>
<td>10.37</td>
<td>100.85</td>
<td>60.6</td>
<td>100.07</td>
</tr>
<tr>
<td>4.</td>
<td>10.33</td>
<td>99.15</td>
<td>60.76</td>
<td>100.19</td>
</tr>
<tr>
<td>5.</td>
<td>10.36</td>
<td>100.43</td>
<td>60.72</td>
<td>100.16</td>
</tr>
<tr>
<td>6.</td>
<td>10.36</td>
<td>100.43</td>
<td>60.51</td>
<td>100.01</td>
</tr>
<tr>
<td>Mean</td>
<td>10.34</td>
<td>99.71</td>
<td>60.5</td>
<td>100.00</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.02</td>
<td>0.99</td>
<td>0.25</td>
<td>0.185</td>
</tr>
</tbody>
</table>

Limits of detection (LOD) and of quantification (LOQ)

The signal-to-noise ratio of 3.3:1 and 10:1 were considered as LOD and LOQ and were found to be 0.40 and 1.0 ng/ml, respectively.

**Selectivity and specificity**

The results indicated that there was no overlap between the analyte and the rest materials, and the retention time of amitriptyline was found to be 6.15 min. The specificity of the method was shown by the lack of interfering endogenous...
plasma components in the chromatograms of screened plasma batches.

**Stability**

Stability (short-term) was validated for 6 and 48 h, both at room temperature. Long-term stability was evaluated after storage of the test samples at - 80 °C for 30 days. All stability experiments were carried out against freshly spiked calibration standards.

**Analysis of amitriptyline in plasma by the developed method**

The developed HPLC method was successfully used to quantify amitriptyline in plasma samples, following oral administration of drug and activated charcoal. The resulting drug concentrations measured in plasma collected after 2 h of dosing. The concentrations of drug obtained in plasma in all four animal groups are presented in (Table 3). One way analysis of variance (ANOVA) was used to interpret the results obtained after plasma analysis in all groups and the results were presented well in (Table 3). The statistical results are also displayed in Table 4 in terms of degree of freedom, p-value, etc. The statistical size of the effect was also be calculated as in Eq 1.

\[ r^2 = \eta^2 = \frac{SS_{\text{among group}}}{SS_{\text{total}}} = 3949.375/6485.220 = 0.609 \ldots \ldots \ldots (1) \]

This value \( r^2 = 0.609 \) can also be explained on the basis of the guidelines by Cohen, which stipulates that 0.1, 0.25 and 0.4 represents small, medium and large effect sizes, respectively. Therefore, it is evident from the \( r^2 \) value that it is a size of a large impact. Student-Newman-Keuls method was used to identify the sources of these differences for multiple comparison tests, and the results are shown in Table 5.

The results of statistics indicated that there were significant differences between the average concentration of amitriptyline without the use of activated charcoal, and the average concentration of amitriptyline when the activated charcoal concentrations i.e. 200 mg/ml, 100 mg/ml respectively, were used. Fig. 2 illustrates the differences between the arithmetic mean concentrations of the drug.

**Table 3:** Drug concentrations in blood sample following administration of 2.5 mg mitriptyline to rats

<table>
<thead>
<tr>
<th>Amitriptyline (2.5 mg)</th>
<th>Blood drug concentration (ng/ml, n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I</td>
<td>Group-II</td>
</tr>
<tr>
<td>41.2</td>
<td>36.5</td>
</tr>
<tr>
<td>42.9</td>
<td>44.3</td>
</tr>
<tr>
<td>23.0</td>
<td>39.4</td>
</tr>
<tr>
<td>24.9</td>
<td>39.8</td>
</tr>
<tr>
<td>23.5</td>
<td>22.0</td>
</tr>
<tr>
<td>52.9</td>
<td>51.2</td>
</tr>
<tr>
<td>25.7</td>
<td>30.8</td>
</tr>
<tr>
<td>58.8</td>
<td>36.1</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>36.61 ± 14.30</td>
</tr>
</tbody>
</table>

**Table 4:** Results of statistical analysis

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>DF</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>3</td>
<td>3949.375</td>
<td>1316.458</td>
<td>14.536</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Residual</td>
<td>28</td>
<td>2535.845</td>
<td>90.566</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>31</td>
<td>6485.220</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5:** Student-Newman-Keuls Method for all pair-wise multiple comparison procedures

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Differences of means</th>
<th>P</th>
<th>q</th>
<th>p</th>
<th>P &lt; 0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Row 2 vs. Row 4</td>
<td>25.550</td>
<td>4</td>
<td>7.594</td>
<td>&lt; 0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Row 2 vs. Row 3</td>
<td>18.700</td>
<td>3</td>
<td>5.558</td>
<td>&lt; 0.002</td>
<td>Yes</td>
</tr>
<tr>
<td>Row 2 vs. Row 1</td>
<td>0.900</td>
<td>2</td>
<td>0.267</td>
<td>&lt; 0.851</td>
<td>No</td>
</tr>
<tr>
<td>Row 1 vs. Row 4</td>
<td>24.650</td>
<td>3</td>
<td>7.326</td>
<td>&lt; 0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Row 1 vs. Row 3</td>
<td>17.800</td>
<td>2</td>
<td>5.290</td>
<td>&lt; 0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Row 3 vs. Row 4</td>
<td>6.850</td>
<td>2</td>
<td>2.036</td>
<td>&lt; 0.161</td>
<td>No</td>
</tr>
</tbody>
</table>
It is clear from the above plot that there is no statistical difference between the mean concentration of amitriptyline when 100 and 200 mg/ml activated charcoal were used.

DISCUSSION

In the present study, LOD and LOQ were 0.40 and 1.0 ng/ml, respectively, which indicate an adequate sensitivity of the developed method. Selectivity was performed to differentiate amitriptyline from rest of the material present in the sample and to determine the retention time of amitriptyline. The retention time of the amitriptyline was 6.15 min in all samples, which means that there was no overlap between the analyte and the rest materials. The stability experiments results were always well within ± 15 % of deviation, and thus indicates that no degradation of amitriptyline occurred under the defined conditions.

The results of the analysis indicated that, using the half of the standard dose (100 mg/ml) of the activated charcoal gave comparable effect to that of the standard dose (200 mg/ml) in reducing the concentration of amitriptyline in the blood as evidenced by the plasma concentration of drug as indicated in (Table 3). On the other hand, using quarter of the standard dose (50 mg/ml) of activated charcoal did not produce a significant effect in reducing the drug concentration in blood after 2 h of dosing.

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (p < 0.001). Since the level of significance calculated (p < 0.001) is smaller than the significance level of statistical study (0.05), there was no significant differences for the average concentrations of amitriptyline between the control group and the other two groups of focus those were given 200 mg/ml and 100 mg/ml of activated charcoal (Table 4).

There was statistically significant difference between the mean concentration of amitriptyline when 50 and 200 mg/ml activated charcoal concentration was used; same was observed when 50 and 100 mg/ml activated charcoal concentration was used. However, no statistically significant difference was found when 100 and 200 mg/ml activated charcoal concentrations were used. No statistically significant differences between the average concentration of Amitriptyline without the use of activated charcoal, and the average concentration of Amitriptyline when 50 mg/ml of activated charcoal concentration was used.

It was also observed that a small dose of activated charcoal (100 mg/ml) produced an effect that was comparable to that of the highest standard dose of the activated charcoal (200 mg/ml) in terms of reducing the toxic concentration of amitriptyline in the blood of rats. We also note that the use of half of the minimum dose of the standard dose of the activated charcoal, 50 mg/ml had a very limited effect in reducing the toxic concentration of the drug.
CONCLUSION

The developed RP-HPLC method enables determination of amitriptyline with good separation and resolution of the chromatographic peaks. Furthermore, using half of the standard dose of activated charcoal produced a comparable effect to the standard dose in reducing blood drug concentration. The method is simple and accurate, and may be considered for routine analysis of amitriptyline in biological samples, raw materials and pharmaceutical formulations, as well as for therapeutic drug monitoring in clinical practice.

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