Original Research Article

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 KH_2PO_4 buffer (60: 40, v/v) solvent system on an Xterna® ms C18 SUM column (5 µm, 3.9 × 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, $r^2 = 0.996$, 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 - 1.0g/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. Xterna® ms C18 SUM (5 μm, 3.9 × 150 mm) column was utilized to elute the compounds of interest at a λ_{max} = 293 nm for the analysis of amitriptyline.

Selection of mobile phase

Different combinations of solvent systems of acetonitrile, buffer (KH_2PO_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_2PO_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 ºC 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 ºC against fresh standard solutions. Long-term stability was evaluated after storing samples at –80 ºC 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 after ten minutes 1 ml of activated charcoal (50 mg/ml, i.e., quarter dose of the standard dose of the activated charcoal) by oral route. Group III was administered 1 ml of amitriptyline (2.5 mg/ml) and after ten minutes 1 ml of activated charcoal (100 mg/ml, i.e., half dose of the standard dose of the activated charcoal). Group IV served as standard control and was given 1 ml of amitriptyline (2.5 mg/ml) and after ten min,
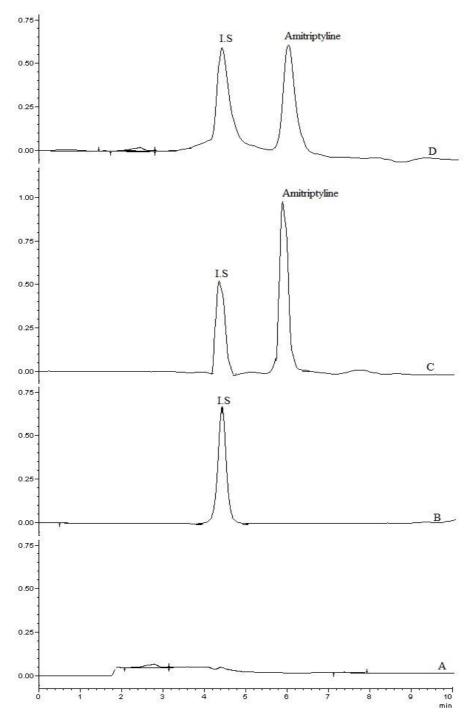
Figure 1: HPLC chromatogram of (A) blank plasma, (B) plasma spiked with internal standard (I.S), (C) plasma sample from the calibration curve of amitriptyline (20 ng/ml; Rt, 6.15 min) and I.S (Rt 4.5 min), and (D) plasma sample from rat administered with amitriptyline (2.5 mg/kg, p.o)

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 anesthetized and blood was collected from heart. Plasma was separated by centrifugation at 2500 × 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 ortho-phosphoric 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 1: Concentration of amitriptyline (30 ng/ml) injected and mean recovery

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

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 %.

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

Table 2: Recovery of the developed HPLC method (n = 6)

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

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 ºC 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.

$$r^2 = \eta^2 = SS_{amonggroup}/SS_{total} = 3949.375/6485.220 = 0.609 \ldots\ldots(1)$$

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

Amitriptyline (2.5 mg)	Blood drug concentration (ng/ml, n = 8)			
	Group-I	Group-II	Group-III	Group-IV
	41.2	36.5	35.5	19.6
	42.9	44.3	20.4	13.8
	23.0	39.4	14.4	13.0
	24.9	39.8	16.8	13.1
	23.5	22.0	15.3	9.3
	52.9	51.2	19.9	13.6
	25.7	30.8	9.0	2.5
	58.8	36.1	18.9	10.8
Mean ± SD	36.61 ± 14.30	37.51 ± 8.71	18.81 ± 7.64	11.96 ± 4.85

Table 4: Results of statistical analysis

Source of variation	DF	Sum of squares	Mean square	*F*-value	*P*-value
Between Groups	3	3949.375	1316.458	14.536	< 0.001
Residual	28	2535.845	90.566		
Total	*31*	*6485.220*			

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

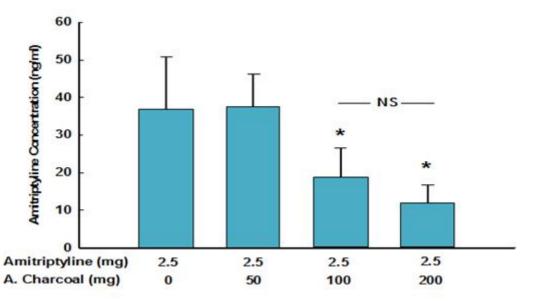| Comparison	Differences of means	P	q	*p*	*P < 0.050*
Row 2 vs. Row 4	25.550	4	7.594	*< 0.001*	Yes
Row 2 vs. Row 3	18.700	3	5.558	*< 0.002*	Yes
Row 2 vs. Row 1	0.900	2	0.267	*< 0.851*	No
Row 1 vs. Row 4	24.650	3	7.326	*< 0.001*	Yes
Row 1 vs. Row 3	17.800	2	5.290	*< 0.001*	Yes
Row 3 vs. Row 4	6.850	2	2.036	*< 0.161*	No

Figure 2: Statistical graph explains the differences between the arithmetic mean concentrations; *p <0.05, **p <0.01, ***p < 0.001

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.

ACKNOWLEDGEMENT

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