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High-performance liquid chromatographic determination of quinine in rat biological fluids

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Abstract

A high-performance liquid chromatographic (HPLC) method with ultraviolet detection for the determination of quinine in rat biological fluids is described. Due to its selectivity and sensitivity, the proposed method can be used in the case of such rat biological fluids as cerebrospinal fluid (CSF) and perilymph for which the accessible volumes are limited to 100 μl and 10 μl, respectively. Consequently, the assay method has been applied to the measurements of quinine concentration in rat plasma, CSF and perilymph samples.

Keywords: Quinine

1. Introduction

The cinchona alkaloid quinine has been widely used for the treatment of malaria for centuries (since 1633). It has recently been reported that quinine (after salicylate) is the next drug used to induce tinnitus. Moreover, it has been found that this drug is able to induce tinnitus in humans without significant side effects [1–3]. Tinnitus, commonly known as ringing in the ears, is estimated to profoundly affect the lives of a large part of the human population, e.g. about 9 million Americans [1,4].

An animal model of tinnitus in which sodium salicylate [5–8], as well as quinine [9] was used to induce this phenomenon in rats has been proposed. In such models, it is necessary to know the time course of drugs used for inducing tinnitus, in rat biological fluids. In the case of salicylate ions the spectrophotometrical analytical method [10] for the determination of the concentration of this species, in serum and other biological fluids, is well known and frequently used.

For quinine, one can observe the continuous development of rapid, sensitive and selective methods for the determination of this drug in biological fluids. Non-chromatographic methods, which quantify both quinine and its diastereoisomer quinidine, have been available since 1943 [11]. The most widely used extraction procedure is the method of Cramer and Isaksson [12] with fluorescence measurements. Other non-specific colorimetric and fluorometric methods have also been used to quantify quinine and its metabolites in biological fluids [13–15]. However, the above-mentioned techniques lack selectivity and sensitivity [16–18]. These limitations have been overcome by high-performance liquid chromatographic (HPLC) methods [16–20], which enable quinine or/and its isomers to be measured with accurate separation and quantification of metabolites [19]. A comparison between the non-chro-

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matographic [12] and HPLC methods [18] showed that the non-chromatographic values of quinine concentration were on average almost 40% higher than those obtained using HPLC. This difference is due to the contribution of quinine metabolites in biological fluids when non-chromatographic methods are used. Therefore, more specific HPLC techniques have recently been developed using complicated extraction procedures and reversed-phase or ion-pair chromatography [16–20].

There are several HPLC methods which enable quantification of quinine. For instance, Epstein et al. [18] developed a simple, rapid and accurate HPLC procedure for the estimation of quinine in human serum. However, quinidine – the diastereoisomer of quinine – has a retention time similar to that of quinine, and thus interferes with quinine assays when using the Epstein procedure. Recently Mihaly et al. [16] described an HPLC method which allows both quinine and quinidine to be measured concurrently. However, most of the above-mentioned HPLC methods require large-volume plasma samples, and thus are not suitable for continuous drug monitoring in rat plasma. Probably for the same reason, the above-mentioned HPLC procedures have not been applied for other biological fluids such as cerebrospinal fluid (CSF) and perilymph. In the case of these studies it is of great importance to know the range of quinine concentrations in plasma, CSF, and perilymph. It would be helpful in understanding the mechanism of tinnitus in rats.

The aim of this work was therefore, to develop a rapid, selective and highly sensitive method for the determination of quinine level in rat biological fluids. The latter factor is of great importance due to the very limited volume of rat perilymph (in the range of 10 μl). The accessible CSF volume is also limited, usually below 100 μl. The assay method has been applied to the measurements of rat plasma, CSF and perilymph samples.

2. Experimental

2.1. Conditions

HPLC assays were carried out on a Beckman System Gold liquid chromatographic system (Beckman Instruments, Columbia, MD, USA) which consisted of a solvent delivery module (Model 110B), a universal 210A injector and a programmable UV detector module (Model 166) at 254 nm. The output from the detector was connected to a 427 integrator module. The system was controlled by PC 8300 NEC controller.

A reversed-phase Rad-Pak μBondapak 18 column (10 μm particles, 100 mm×8 mm I.D, Waters, Rockville, MD, USA) was used. An in-line filter and precolumn (μBondpak C_{18}, 10 μm particles, 30 mm×8 mm I.D, Waters) were used to protect the analytical column.

The mobile phase consisted of water–acetonitrile (91:9, v/v) containing about 1% of triethylamine (to 3000 ml of mobile phase 30 ml of amine was added) was adjusted to pH 2.5 with concentrated orthophosphoric acid (the value of pH measured on the final mobile phase). The mobile phase prepared was subsequently filtrated and de-gassed (using GS type filter, pore size 45 μm, about 30 min) under vacuum. Measurements were carried out at a flow-rate of 3.5 ml min^{-1} (backpressure approximately 400 psi) at ambient temperature.

2.2. Chemicals

Quinidine (QN) hydrochloride monohydrate and quinine (Q) hydrochloride (containing 1.5 H_2O per mol when assayed) were obtained from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Ammonia solution (A.C.S. reagent-specific gravity 0.8), phosphoric acid (99.99%), triethylamine (99+%) as well as ethyl acetate and hexane (both HPLC grade) were obtained from Aldrich (Milwaukee, WI, USA).

2.3. Extraction procedure

Plasma pretreated by vortex mixing and short centrifugation (60 s) was treated according to the modified Mihaly procedure [16]. To biological fluid samples of 20 μl containing quinidine as internal standard (40 ng in 20 μl of HPLC grade water), were added 25 μl of ammonia solution. This mixture was extracted with 250 μl of extraction mixed solvent (hexane–ethyl acetate, 9:1, v/v) applying multi-tube

vortex mixing for 60 s. The extraction was followed by centrifugation at 1000 *g* for 10 min. The known part of the separated organic phase (in the range 300–500 μl) was taken and evaporated using nitrogen evaporator at 35°C. The residue was dissolved in 20 μl of mobile phase and 5 μl of this was injected onto the chromatograph.

In the case of perilymph the accessible volume (the average measured volume of order of 10 μl) was supplemented to 20 μl using saline and the obtained solution was treated in the manner described above.

For CSF (as well as plasma samples taken 24 h after injection when very low concentrations of quinine were expected) double volumes of samples were taken to increase the sensitivity of assay.

In the case of 'macro' plasma samples (volume of samples greater than 20 μl) the same procedure of extraction was applied using recalculated, relatively larger volumes of ammonia solution and extraction mixture (for instance for samples of 100 μl the volumes of ammonia and extraction mixture were 100 μl and 1 ml, respectively).

2.4. Calibration procedure

Calibration lines were prepared by adding a known amount of quinine (in 20 μl of HPLC grade water) to a fixed concentration of internal standard—quinidine (40 ng in 40 μl of HPLC grade water) in drug-free plasma (20 μl). Fourteen quinine solutions of various concentration in the range 0.1–10 μg ml^{-1} (i.e. 0.1; 0.3; 0.5; 0.7; 0.8; 0.9; 1.0; 2.0; 3.0; 4.0; 5.0; 7.0; 9.0 and 10.0 μg ml^{-1}) and constant quinidine concentration (2.0 μg ml^{-1}) were prepared and analyzed in the same way as described above for biological samples. The peak area ratios of drug to internal standard were plotted against the corresponding drug–internal standard weight ratio.

2.5. Analytical recovery

The analytical recoveries of quinine and quinidine were calculated by comparing the peak areas obtained from an extracted plasma containing known amounts of the substances with the peak areas obtained from a direct injection of the same amounts (2 μl ml^{-1}) of each compound. (The direct injections of the mobile phase were made before the determination of the calibration line to check the purity of the used portion of mobile phase).

2.6. Assay precision and accuracy

System precision (intra-run), method precision (inter-run), as well as intra- and inter-run accuracy were determined for several quinine concentrations over the entire calibration range (i.e. 100–10 000 ng ml^{-1}) by replicate assays of spiked plasma samples. The measurements were performed for various volumes of plasma (in the range 20–200 μl). Six samples of the same known concentration of quinine were treated as unknown to evaluate the intra- and inter-run coefficients of variation as well as accuracy of method. Intra-run precision (i.e. system precision) and accuracy were evaluated for one series of six samples (within the same run, using the same calibration line), whereas inter-run precision (i.e. method precision) and inter-run accuracy were determined for six samples measured during six consecutive days (using different calibration lines). Intra-run and inter-run relative standard deviations (in percentages) were taken as coefficients of variations; i.e. system precision (reproducibility of replicate determinations in a run) and method precision (reproducibility between determinations on separate runs). The relative differences between the experimental concentration and analytical concentrations yielded the relative intra- and inter-run errors (i.e. intra- and inter-run method accuracy).

Intra- and inter-run precision was also determined for several biological samples in the relatively wide range of quinine concentrations (0.47–2.06 μg ml^{-1} as given in Table 5) in the same way as for spiked plasma samples. This means that in six plasma samples the concentration of quinine was determined to evaluate relative standard deviations (i.e. system and method precision).

2.7. Study of chronically cannulated rats

The assay was applied to a pilot study in which the level of quinine in rat plasma was measured over three consecutive days. A cannula was inserted into the femoral artery of rats as described elsewhere [21] and the animals were then allowed to recover for

three days prior to experiments. During the next three days quinine (200 mg kg^{-1}) was administered to the rats subcutaneously. Blood samples were taken from the femoral artery pre-dose and at 30; 45; 60; 90; 120; 180 and 240 min after the end of drug infusion. CSF and perilymph samples were taken after one, two and three days of quinine administration. After centrifugation, plasma CSF and perilymph samples were stored at −20°C until assayed for quinine.

3. Results and discussion

The retention times of quinidine (internal standard) and quinine were found to be 6 and 7 min, respectively, i.e. different enough to obtain the complete separation of peaks. The chromatogram of blank plasma was free from all chromatographic interference and both compounds were completely resolved to baseline. Under the conditions applied all the quinine metabolites would be expected to elute with or near the void volume [19].

Calibration lines prepared on eleven different days were linear in the quinine concentration range 100–10 000 ng ml^{-1} with correlation coefficients of the order of 0.9992. The calibration line in the range of interest for this study is represented by Eq. (1):

$$R = b \cdot S + a \quad (1)$$

where $R = m_Q/m_{QN}$ denotes quinine–quinidine weight ratio and $S = S_Q/S_{QN}$ denotes quinine–quinidine peak area ratio. The parameters a and b of calibration lines together with the values of their standard deviation, as well as the correlation coefficients k for each calibration day are collected in Table 1.

The minimum detectable level (defined as a peak four times that of baseline noise) was 10 ng/ml of serum (0.05 ng per 5 μl injected) on the detector sensitivity used (0.005 AUFS). The average recovery of quinine and quinidine from plasma was 71.5% and 70%, respectively. (Recovery of quinine varied in the range 67–76%, while that for quinidine from 54–86% for all samples studied over the entire range of concentration). However, due to the very similar behaviour of both substances under study during extraction (i.e. the quinine/quinidine recovery ratio was pretty stable for all samples studied), changes in recovery were of minor importance. On the basis of this observation we assumed that the quinine/quinidine recovery ratios from perilymph and CSF are of the same order as from plasma.

Table 1
Comparison of the parameters of calibration lines determined on eleven subsequent days of measurements

Day no.	Slope (b)	Intercept (a)	Correlation coefficient (k)
1	0.488(0.005)	0.007(0.012)	0.9993
2	0.487(0.007)	0.017(0.016)	0.9988
3	0.491(0.005)	−0.008(0.012)	0.9993
4	0.487(0.005)	0.007(0.012)	0.9993
5	0.490(0.005)	0.001(0.011)	0.9994
6	0.491(0.006)	−0.001(0.013)	0.9992
7	0.488(0.005)	0.011(0.012)	0.9993
8	0.488(0.007)	0.010(0.017)	0.9987
9	0.488(0.007)	0.004(0.017)	0.9987
10	0.487(0.005)	0.002(0.011)	0.9994
11	0.491(0.006)	0.002(0.014)	0.9991

The values of the standard deviation of slope and intercept are given in parentheses.

At the very beginning of these studies, to establish the influence of rat plasma volume on the intra-run precision and accuracy of the method under study, these parameters were determined for four different volumes of spiked plasma samples using relatively low quinine concentrations (below 1 μl ml^{-1}). The results were compared in Table 2. As shown, the relative standard deviations and errors increase with decreased volume of sample being acceptable (i.e below 10%) for volumes as low as 20 μl. It can thus be concluded that in our method even samples as small as 20 μl can be used. It is of great importance, especially in the case of CSF and perilymph when the volume of biological fluid is greatly limited.

Table 2
Comparison of intra-run method precision and accuracy for various volumes of spiked plasma samples

Volume of plasma (μl)	Concentration of spiked plasma (μg ml^{-1})	Relative standard deviations (%)	Relative errors (%)
20	0.60	4.4	4.8
25	0.90	3.5	4.9
100	0.75	2.3	3.6
200	0.625	2.2	0.2

The number of replicates $n = 6$.

The results of evaluation of the precision and accuracy of the 'micro' method (20 μl) for spiked plasma samples (two concentrations of quinine equal to 0.6 and 3.4 μg ml^{-1} were taken for comparison) are summarized in Table 3. They show good precision and accuracy of assays made. The intra-run coefficients of variation were less than 4.4% for both concentrations used, the inter-run variation being less than 6.5% in the applied range of concentration. The relative errors (responsible for the accuracy of method) were less than 4.8% and 4.0% for intra- and inter-run assays, respectively. Additional measurements of intra-run precision for a concentration of quinine as low as 0.4 μg ml^{-1} also indicated sufficient precision of the method (9.7%, i.e. below 10% – see Table 3). It should be noted that the relative standard deviations and errors increase, with decreased concentration of quinine in the sample being acceptable even for concentrations as low as 0.4 μg ml^{-1}.

For comparison, Table 4 shows the results of quinine concentration determination for greater (100 μl) volumes of rat spiked plasma ('macro' method). As can be seen the intra-run (two samples of quinine concentration equal to 0.75 and 1.0 μg ml^{-1} were used), as well as inter-run (two quinine concentrations; 0.6 and 1.5 μg ml^{-1} were applied) precision and accuracy of the method is comparable (being of the same range of magnitude) with that when using 'micro' (20 μl) samples.

Table 5 gives a comparison of the intra- and inter-run precision of the method for four different real plasma samples (after injection of quinine). As can be seen from the data in Table 5, the relative standard deviations increase with decreased concentration of quinine in the sample being acceptable (i.e. below 10%) even for the smallest concentrations measured (samples taken 24 h after injection; the concentration of quinine of the order of 0.5 μg ml^{-1}). The precision of the system (intra-run) is as high as 5% and the precision of the method (inter-run) is also sufficient (9.6%).

As mentioned in the Introduction, the method studied has been used in the analysis of quinine in rat biological fluids; plasma, CSF and perilymph. Peak plasma concentrations of quinine occur within 0.5–3 h after injection. On the third day of repeated administration of 200 mg ml^{-1} drug dose, the maximum drug concentration in the plasma is of the order of 10 μg ml^{-1}. After 24 h no quinine or only a negligible concentration is detectable. An example of the time course of quinine distribution in rat plasma (during the third day of drug dosage) is shown in Fig. 1.

Table 3
Intra-run and inter-run precision and accuracy data for quinine analysis in rat spiked plasma samples (20 μl)

Concentration of spiked plasma (μg ml^{-1})	Concentration measured (μg ml^{-1})	Relative standard deviations (%)	Relative errors (%)
Intra-run data			
0.40	0.41	9.7	2.6
0.60	0.63	4.4	4.8
3.40	3.36	2.1	1.2
Inter-run data			
0.60	0.61	6.5	1.7
3.40	3.26	3.4	4.0

The number of replicates $n=6$.

Table 4
Intra-run and inter-run precision and accuracy data for quinine analysis in rat spiked plasma samples (100 μl)

Concentration of spiked plasma (μg ml^{-1})	Concentration measured (μg ml^{-1})	Relative standard deviations (%)	Relative errors (%)
Intra-run data			
0.75	0.72	2.3	3.6
1.00	0.97	6.6	3.4
Inter-run data			
0.60	0.56	7.5	5.9
1.50	1.50	8.0	0.3

The number of replicates $n=6$.

Table 5
Intra-run and inter-run precision data for quinine analysis in biological plasma samples (20 μl)

Sample	Concentration measured (μg ml^{-1})	Relative standard deviations (%)
Intra-run data		
1	0.47	5.0
2	2.17	3.4
Inter-run data		
1	0.53	9.6
2	2.06	4.9

The number of replicates $n=6$

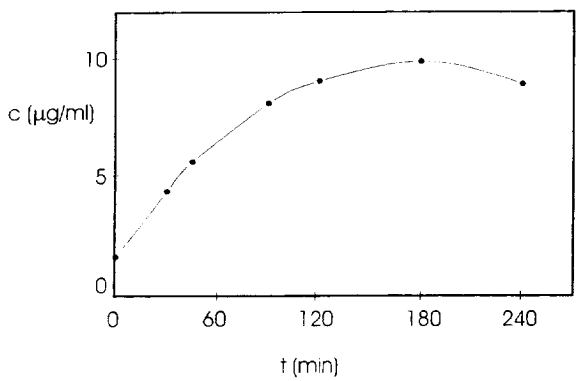

Fig. 1. Example of time course of quinine distribution in rat plasma.

In the case of CSF, the concentration of quinine is in the range of 0.1–0.6 μg ml^{-1}. (The concentration of the alkaloid is only 3–8% of that in the plasma). For the sake of testing the precision and accuracy of method under study for CSF measurements, specially designed samples were run. They consisted of plasma samples spiked by quinine to the same concentration as the average level found in CSF (0.2 μg ml^{-1}). Obtained in this way the inter-assay precision and accuracy parameters (the relative standard deviations and the relative errors, respectively) were 12.4% and 8.7% for 20 μl samples, 6.2% and 9.6% for double volume (2×20 μl) of samples and 2.4% and 7.3% for triple volume (3×20 μl) of plasma samples spiked by quinine to the same concentration as level found in CSF, respectively. This means, that even for the lowest quinine concentration measured in CSF the precision and accuracy of the method under study is sufficient enough (below 10%) if at least double volume (with respect to 20 μl) of CSF sample is taken for measurement.

For perilymph the concentration of quinine is in the 0.3–0.9 μg ml^{-1} range, being about twice as high as in CSF. Using the same approach as in the case of CSF the precision and accuracy of method used was tested using especially designed samples, i.e. plasma spiked by quinine to the same concentration as the level found in perilymph. A test was performed for the average concentrations found in the perilymph (0.6 μg ml^{-1}) and the lower limit of average range of accessible perilymph volume (8 μl). The obtained values of intra-run coefficient and relative error were 9.0% and 5.4%, respectively, which indicate acceptable precision and accuracy of the method under study.

In summary, a rapid, selective and sensitive method for the determination of quinine levels in rat plasma, CSF and perilymph has been demonstrated. The method described is sufficiently selective and sensitive to be used in studies concerning the mechanism of tinnitus in rats.

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