Simultaneous Quantitation of the Highly Lipophilic Atovaquone and Hydrophilic Strong Basic Proguanil and Its Metabolites Using a New Mixed-Mode SPE Approach and Steep-Gradient LC

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Abstract

A bioanalytical method is described for the simultaneous quantitative analysis of the highly lipophilic atovaquone and the strong basic proguanil with metabolites in plasma. The drugs are extracted from protein precipitated plasma samples on a novel mixed-mode solid-phase extraction (SPE) column containing carboxypropyl and octyl silica as functional groups. The analytes are further separated and quantitated using a steep-gradient liquid chromatograhic method on a Zorbax SB-CN column with UV detection at 245 nm. Two different internal standards (IS) are used in the method to compensate for both types of analytes. A structurally similar IS to atovaquone is added with acetonitrile to precipitate proteins from plasma. A structurally similar IS to proguanil and its metabolites is added with phosphate buffer before samples are loaded onto the SPE columns. A single elution step is sufficient to elute all analytes. The method is validated according to published guidelines and shows excellent performance. The within-day precisions, expressed as relative standard deviation, are lower than 5% for all analytes at three tested concentrations within the calibration range. The between-day precisions are lower than 13% for all analytes at the same tested concentrations. The limit of quantitation is 25nM for the basic substances and 50nM for atovaquone. Several considerations regarding development and optimization of a method for determination of analytes with such a difference in physiochemical properties are discussed.

Introduction

Malaria is one of the oldest and most pervasive diseases, with half of the world's population still encountering Plasmodium. The increasing resistance of malaria parasites to antimalarial compounds have contributed to a resurgence of malaria. The World Health Organization has estimated that more than 400 million fall ill and between 1 and 3 million die anually (1).

Atovaquone (ATQ) is a highly lipophilic antimalarial compound that is extensively bound (> 99.9%) to plasma proteins (2,3). The compound was developed as an antimalarial drug but has also shown to be effective in the treatment of AIDS patients (4). The combination (Malarone) of ATQ and another antimalarial compound, proguanil (PG), has shown to be very effective against severe falciparum malaria (5,6). However, the use of Malarone that is unprotected by other drugs (such as artemisinin derivates) in endemic areas where it is not needed will encourage the emergence of resistance (7). The determination of drug levels in blood is a predictive tool for assessing the patient's compliance with chemoprophylaxis, establishing whether a therapeutic concentration has been obtained, or suggesting the presence of a resistant strain of the parasite. The first evidence of in vivo resistance to treatment with Malarone was recently reported (8). Because of resistance shown at an early stage, there is an urgent need for careful surveillance of the efficacy and evaluation of combinations with new protective drug partners. Further evaluation of methods that allows simultaneous determination of ATQ, PG, metabolites, and, possibly, other antimalarial drugs is essential. PG is only 75% protein bound, and the in vitro binding is unaffected by therapeutic concentrations of ATQ (9). PG acts as a prodrug, activated by the hepatic cytochrome P-450 isoenzyme to its active metabolite cycloguanil (CG) and the less active metabolite 4-chlorophenylbiguanid (4-CPB) (10,11). The enzyme P-450 2C19 exhibits genetic polymorphism, leading to big differences in the amount of metabolites formed between poor and extensive metabolizers (9).

Several clinical trials for malaria treatment/prophylaxis have been conducted for Malarone, and typical, steady-state plasma concentrations of ATQ are approximately 8–26µM. Steady-state plasma concentrations of PG and CG are in the range of 260–830 and 6–360nM, respectively (12).

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Several methods have been reported for the analysis of PG and its metabolites in plasma and for the analysis of ATQ in plasma (13–21). Two methods have allowed simultaneous liquid chromatographic (LC) separation of ATQ, PG, and metabolites (22,23). There has been no report of a method for the simultaneous determination of ATQ, PG, and its metabolites in plasma or any other biological fluid.

Developing a method for simultaneous determination of ATQ, PG, and its metabolites in plasma is a great challenge because their physicochemical properties differ significantly. Atovaquone is a highly lipophilic weak acid with a log D that is approximately 5.5 below pH 5. Proguanil and its metabolites are very strong bases that have a log D of approximately –4 below pH 7, pK_{a1} of 12.6, and pK_{a2} of 10.6 (24).

Conventional mixed-mode columns contain a strong cation exchanger and C8/C18 phase and have been used for extreme purification of basic compounds from biological samples, especially for drugs of abuse (25). Mixed-mode columns have also been used within toxicological drug screening to simultaneously determine a variety of neutral, acidic, and basic drugs (26–34). These methods have often used sequential elution in fractions or different sample preparations for the different classes. However, extraction conditions for extremely hydrophobic compounds and very strong basic compounds have not been studied extensively. The methods have not been further optimized with respect to accuracy and precision for any particular compound, but they merely acted as a general procedure for screening of as wide a range of analytes as possible.

A new type of mixed-mode column HCX-Q, containing weak cation exchanger and octyl silica as functional groups, is suitable for simultaneous analysis of highly lipophilic and strong basic compounds. Good quantitative reproducibility for the analytes has been the key response rather than absolute recovery during method development work. As so, solubility of the lipophilic ATQ has been extensively studied and often forced minimum requirements on the solution in the different method steps. A conventional mixed-mode column using a sulfonic ion exchanger was unsuitable because the polybasic analytes bound too strong to the column and eluted with a low, nonreproducible recovery. This has also been shown by the solid-phase extraction (SPE) column manufacturer for PG with metabolites and the quaternary amine paraquat (35). The novel mixed-mode column contains a weak cation exchanger, which can be made uncharged in the elution step instead of the basic analytes. A recently published paper compared different SPE columns (including mixed mode) for screening in toxicological analysis and suggested a C8 column to be most suitable (36). The new mixed-mode column could probably yield even higher recoveries because of the additional weak cation exchanger part, which could contribute to weak ion–ion interactions.

The aim of this work was to develop and validate an automated SPE–LC method for the simultaneous determination of ATQ, PG, CG, and 4-CPB in plasma. The assay has been validated with respect to accuracy, precision, linearity, selectivity, stability, and recovery, according to published guidelines (37,38).

Experimental

Chemicals and materials

ATQ and ATQ–internal standard (IS) were obtained from Glaxo Wellcome (Hertfordshire, U.K.). PG, CG, 4-CPB, and CG-IS were obtained from Zeneca (Cheshire, U.K.). The structures are shown in Figure 1. Acetonitrile (ACN) and methanol were of high-performance LC grade (CHROMASOLV) from Sigma (St. Louis, MO) and dimethylformamide [pro analysi grade (p.a.)], ortho-phosphoric acid (p.a.), and sodium hydroxide (p.a.) were obtained from VWR International (Darmstadt, Germany).

The water was purified using a Milli-Q Academic system (Millipore, Bedford, MA). The phosphate buffer solutions were prepared by mixing different amounts of 1.0M ortho-phosphoric acid and 1.0M sodium hydroxide with Milli-Q water.

Instrumentation and chromatographic conditions

The chromatographic (LC) system consisted of a SP-8810 LC pump, SP 8880 autosampler, and spectra system UV 2000 detector set at 245 nm. All instruments were from Spectra Physics (San José, CA).

Data acquisition was performed using Chromatography station for Windows 1.7 (DataApex Ltd., Prague, Czech Republic). The mobile phases were: (A) ACN–phosphate buffer, (pH 2.6, 0.1M) (22:78 v/v), (B) ACN–phosphate buffer (pH 2.6, 0.03M) (60:40 v/v), and (C) ACN–phosphate buffer (pH 2.6, 0.03M) (80:20 v/v). The analytes were eluted with mobile phase 100% A between 0 and 9 min, switching from 100% A to 100% B between 9.0 and 9.5 min, 100% B between 9.5 and 22 min, switching from 100% B to 100% C between 22.0 and 22.2 min, 100% C between 22.2 and 24.5 min, and equilibrated again with 100% A between 25 and 35 min. A flow rate of 1.0 mL/min was used through a Zorbax SB-CN, (250- × 4.6-mm i.d., 5 µm), (Zorbax, Wilmington, NC), protected by a short guard column SecurityGuard C18 (4 × 3 mm, 5 µm) (Phenomenex, Torrance, CA).

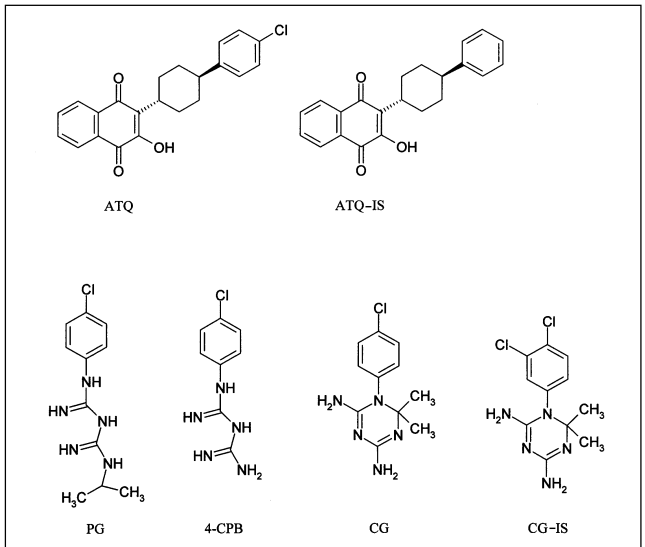

Figure 1. Structures of ATQ, ATQ–IS, PG, 4-CPB, CG, and CG–IS.

SPE

Extraction was carried out on an automated SPE system, ASPEC XL (Gilson, Middleton, WI) using a novel mixed-mode extraction column (HCX-Q) containing carboxypropyl and octyl silica as support (1 mL, 100 mg, Argonaut Ltd., Hengoed, U.K.). The ASPEC system uses positive air pressure instead of vacuum to get the liquids through the columns.

Preparation of calibration standards and samples for determination of accuracy and precision

Concentrated stock solutions of ATQ and ATQ–IS (500µM) were prepared in methanol–dimethylformamide (99:1 v/v). Concentrated stock solutions of PG, CG, 4-CPB (500µM), and CG–IS (100µM) were prepared in acidified deionized water. The solutions were stored at approximately +4°C. Appropriate amounts of the stock solution of ATQ, PG, CG, and 4-CPB were added to blank plasma to yield a spiked calibration curve ranging for ATQ from 50 to 14830nM and for PG, CG, and 4-CPB from 25 to 2000nM. The calibration standards were prepared in batches of 5 mL and stored in 0.5-mL aliquots at approximately –20°C. Calibration curves were generated using seven different concentrations of ATQ, PG, CG, and 4-CPB. The peak-height ratio ATQ to ATQ–IS and PG, CG, and 4-CPB to CG–IS against concentration with nonweighted linear regression was used for quantitation.

Samples for determination of accuracy and precision in plasma were prepared at three concentrations (97, 870, and 11850nM for ATQ and 96, 903, and 1790nM for PG, CG, and 4-CPB) in batches of 10 mL and stored in 0.5-mL aliquots at approximately –20°C.

Sample preparation

All samples were cooled to approximately +4°C prior to protein precipitation. To 500 µL plasma, 1000 µL ATQ–IS (2.5µM) in ACN (ice cold) was added and the tube contents were mixed for 10 s. The tubes were then left to stand for 10 min at room temperature, followed by centrifugation at 13,400 *g* for 10 min. The liquid phase was decanted into new tubes, 1000 µL CG–IS (250 nmol/L) in phosphate buffer (pH 6.8, 0.01M) was added and all of it was loaded onto the SPE column. The extraction procedure for plasma on the ASPEC XL is shown in Table I. The eluates were then evaporated to dryness at 60°C under a gentle stream of air and reconstituted in 40 µL methanol–water (90:10 v/v). Twenty microliters was injected into the LC system.

Solubility and adsorption

The big difference in physiochemical properties of the analytes made it necessary to conduct an extensive evaluation of both adsorption and solubility. Solubility was evaluated by addition of equal amounts of stock solution of the analytes to 14 vials. ACN and water were thereafter added in different amounts to produce a 2-mL sample (duplicates at each ACN level) with final concentrations of ACN in the vials ranging from 10% to 70%. The concentration of the analytes was chosen equal to the highest point in the calibration range. The vials were left undisturbed for approximately 24 h, after which the upper 0.5 mL in one set of vials was transferred to an injector vial. Twenty microliters of each sample was injected into the LC system. The vials from the other set were mixed vigorously precisely before injection. Aliquots of a spiked analyte solution (~ 1000nM) were transferred into vials and inserts of different materials and left undisturbed for approximately 24 h before being injected into the LC system. The solution contained the minimum amount required of organic solvent as determined in the solubility experiment. Spiked plasma samples at a low concentration were processed as described, and the eluates were collected in plastic microtubes, 5-mL plastic tubes, and 5-mL glass tubes. The eluates were evaporated, reconstituted, and injected into the LC system.

Assay validation

The accuracy and precision of the method were estimated by analysis of spiked plasma at three concentrations (97, 870, and 11850nM for ATQ and 96, 903, and 1790nM for PG, CG, and 4-CPB) in triplicate for six analytical runs. Concentrations were determined using a calibration curve prepared on the day of analysis and within- and between-run precisions were calculated. The lower limit of quantitation (LOQ) was selected at the concentration at which the assay precision was within 15% and the signal-to-noise ratio (s/n) exceeded 10:1. The limit of detection (LOD) was determined at the concentration at which the s/n exceeded 3:1.

Calibration curves were constructed using seven calibration standards and were obtained by calculating the peak-height ratios of ATQ to ATQ-IS and PG, CG, and 4-CPB to CG-IS against the corresponding concentration. Linear calibration curves were generated by nonweighted linear regression analysis.

The extraction recovery was determined from the samples used for the precision study. Three concentrations (97, 870, and 11850nM for ATQ and 96, 903, and 1790nM for PG, CG, and 4-CPB) in triplicate together with 9 blank plasma samples were analyzed for 5 days. Prior to evaporation, SPE eluates from blank plasma samples were spiked at the same nominal concentration as the precision samples. Peak-height ratios of the analytes in these spiked extracted blank matrices were compared with those of the precision samples. In this way, any effect of the matrix did not complicate the interpretation of the recovery data (39).

ATQ, PG, CG, and 4-CPB have previously shown good

Table I. ASPEC SPE Procedure for the Extraction of ATQ and Bases from Plasma

Step	Volume	Solvent	Speed
Activation	2 mL	Methanol	3 mL/min
Conditioning	2 mL	ACN–PB (pH 6.8, 0.01M) (40:60 v/v)	3 mL/min
Loading	2.35 mL	Sample	0.5 mL/min
Washing	2 mL	Methanol–PB (pH 6.8, 0.005M) (30:70 v/v)	2 mL/min
Elution	2 mL	Methanol–acetic acid, glacial (98:2 v/v)	0.5 mL/min

stability in plasma and various kinds of buffers and acidic and basic solutions (14,19,21). The stability in the evaporation step was investigated by storing eluates in the heating block for 0, 30, 60, and 120 min after complete evaporation. The samples that were stored for 120 min were first stored at 60°C for 1 h and then at 100°C for the last hour. The other samples were stored at 60°C.

Blank plasma from six healthy volunteers was analyzed, and the chromatograms were examined for endogenous compounds that could interfere with the quantitation of the analytes. Several common antimalarials and some of their metabolites (e.g., amodiaquine, amodiaquine metabolite, chloroquine, chloroquine metabolite, tafenoquine, lumefantrine, piperaquine, pyronaridine, and sulfadoxine) were injected into the LC system to see if they interfered with the peaks of the analytes.

Results and Discussion

ATQ is highly lipophilic and water solubility is less than 300nM (40). The main route of elimination of ATQ is via the liver further to bile and feces and only a small fraction (< 0.6%) is eliminated via the urine because of the extensive degree of protein binding (2,3,40). Earlier published methods for the determination of ATQ have used reversed-phase LC systems with typical mobile phases containing 65–90% organic modifier (14–16,19,21). In comparison, an earlier published method for PG used a cyanopropyl (CN) column with a mobile phase containing 22% ACN and pH 2.6 buffer (13,23). Our initial method for the separation of ATQ, PG, and its metabolites used a mixed-mode LC column but had the drawback of poor peak shape (i.e., wide and fronting) for the basic peaks and, consequently, lower sensitivity (23). An additional problem was that the ion-exchange part in the LC column had limited stability. The initial approach was to use counter ions and pH to achieve separation between ATQ, PG, and metabolites on either a C18 or a CN column. All attempts on the C18 phase were fruitless because it was impossible to separate PG and the metabolites out from the solvent front and still maintain reasonable retention time (i.e., < 60 min) for ATQ. However, separation was successfully achieved on a CN column using ACN–phosphate buffer (pH 7, 0.01M) (53:47 v/v) 0.015M $NaClO_4$ (Figure 2). This method later proved to be unsuitable because extracted blank plasma contained a lot of interfering peaks, two of them coeluting with ATQ (as shown in Figure 3). The next alternative was to evaluate a gradient method on the CN column. The initial conditions were set to 22% ACN, which was the same as the previous method for PG (13). The optimal condition was not to use a continuous gradient but to use a gradient program consisting of three different mobile phases (see Instrumentation). Different ionic strengths and a slightly longer switching time down from 80% to 22% ACN were required to prevent precipitation of phosphate salts in the system. This LC configuration led to severe problems when it came to choosing a reconstitution solvent for the dried SPE eluates. Normally, peak compression is obtained in a reversed-phase LC system if samples are reconstituted in a solution containing less organic modifier than the mobile phase. Approximately 70% organic modifier compared with mobile phase can normally be used without solubility problems, resulting in good peak efficiency. This was, however, not possible for this method because ATQ required at least 40% ACN in the samples to be completely dissolved at the studied concentrations. Samples were initially reconstituted in 250 µL (40% ACN), and 100 µL was injected into the system but with a result of severe band broadening for the early eluting basic peaks. A lower injection volume (~ 20 µL) would lead to significantly less band broadening but also a great loss in sensitivity with a reconstitution volume of 250 µL. The eluates were then reconstituted in only 40 µL of solution, but this significant change in upconcentration of the samples caused

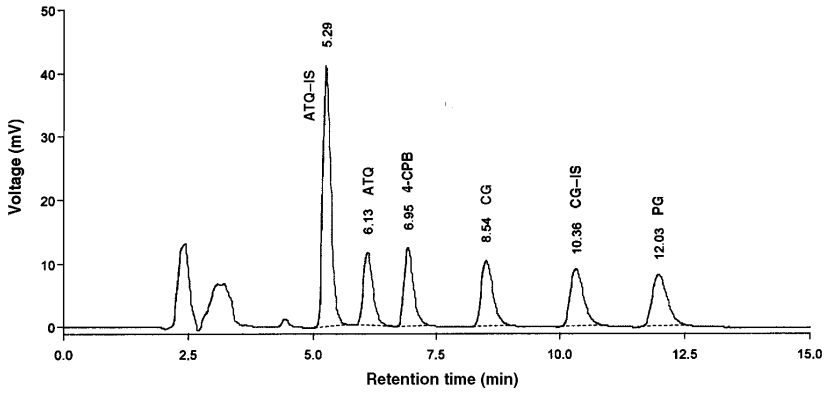

Figure 2. LC separation using CN column with addition of $NaClO_4$ in the mobile phase.

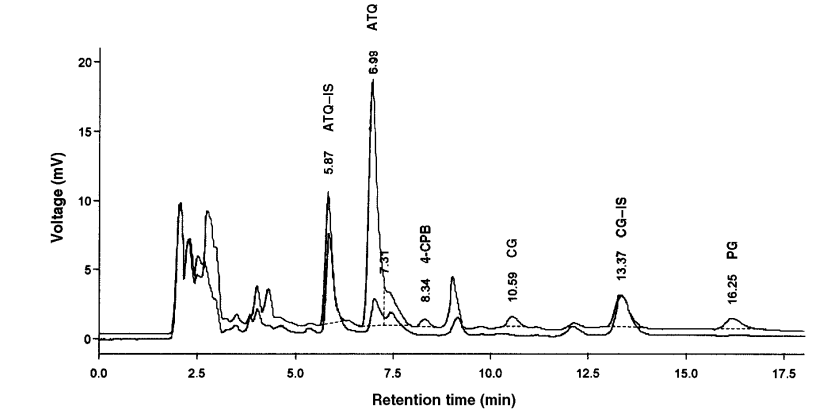

Figure 3. Overlay view of spiked and blank plasma sample.

solubility problems for ATQ at high concentrations and a nonlinear relationship. A linear relationship was obtained when 50% ACN was used in the reconstitution solvent. However, the big difference between the reconstitution solvent (i.e., 50% ACN) and the initial mobile phase (i.e., 22% ACN) caused extensive band broadening for the basic peaks, resulting in poor sensitivity and irreproducible results. By switching to methanol instead of ACN in the reconstitution solvent, it was possible to use 90% solvent and still maintain reasonable peak shape (Figure 4). This is explained by the different solvating properties of methanol compared with ACN, which leads to lower longitudinal diffusion.

Several different elution compositions were evaluated and methanol–acetic acid (98:2 v/v) increased the recovery of ATQ from approximately 70% to nearly 100%. The effect of acetic acid is unclear, but similar results have been reported during liquid–liquid extraction and SPE of the lipophilic drugs lumefantrine (LF) and halofantrine (HFA) (41,42).

Results on solubility and adsorption

The peak heights and areas of each analyte in the 14 vials were compared to evaluate minimum required organic solvent in the samples. There were significantly lower analyte responses for ATQ and ATQ–IS in samples containing less than 40% ACN.

There was also a big difference between the duplicates (i.e., samples injected from the upper 0.5 mL and samples mixed vigorously prior to injection) for ACN levels below 40%. The latter shows the impact that partial solubility would have on the accuracy and reproducibility throughout the LC run (Figure5). Other papers for simultaneous determinations have emphasized the importance of sonication of the samples to disrupt protein bindings prior to SPE (28,29). This is a dangerous approach for analytes with an extensive degree of protein binding. The extent of protein binding has a strong correlation with lipophilicity of the analyte and tend to be almost 100% for very lipophilic drugs (2,3,43–45). Sonication is evidently aiming to disrupt protein bindings and release the analyte as a free solubilized molecule to ensure efficient extraction to the solid phase. However, extremely lipophilic drugs like ATQ, LF, and HFA have extremely limited solubility in a water phase and would need the association with proteins or organic solvents to stay in solution. The recovery of ATQ from plasma in this assay was increased from approximately 10–100% when the proteins were precipitated prior to SPE instead of sample dilution 1:10 with buffer. The low recovery from buffered plasma is a direct result of the very low free fraction of ATQ in plasma. The retention of ATQ on the SPE column is actually reduced approximately 1000 times if loaded in plasma. The retention for a drug in a solution containing proteins loaded onto the SPE column can, with some assumptions, be calculated as:

$$k'_{sp} = k'_s/(1+[PS]/[S]) \qquad \text{Eq. 1}$$

where [PS]/[S] is the drug–protein binding ratio and k'_{sp} and k'_s are the retention with or without proteins in the sample (46). In practice, this means that a drug with 90% protein binding with a theoretical k'_s of 1000 would have a k'_{sp} of 100 in plasma, but a drug with > 99.9% protein binding with a theoretical k'_s of 1000 would have a $k'_{sp} < 1$ in plasma. A higher bed mass would theoretically lead to an increase in k'_s and higher recovery. However, the amount required for drugs with extensive protein binding might turn out to be difficult to use.

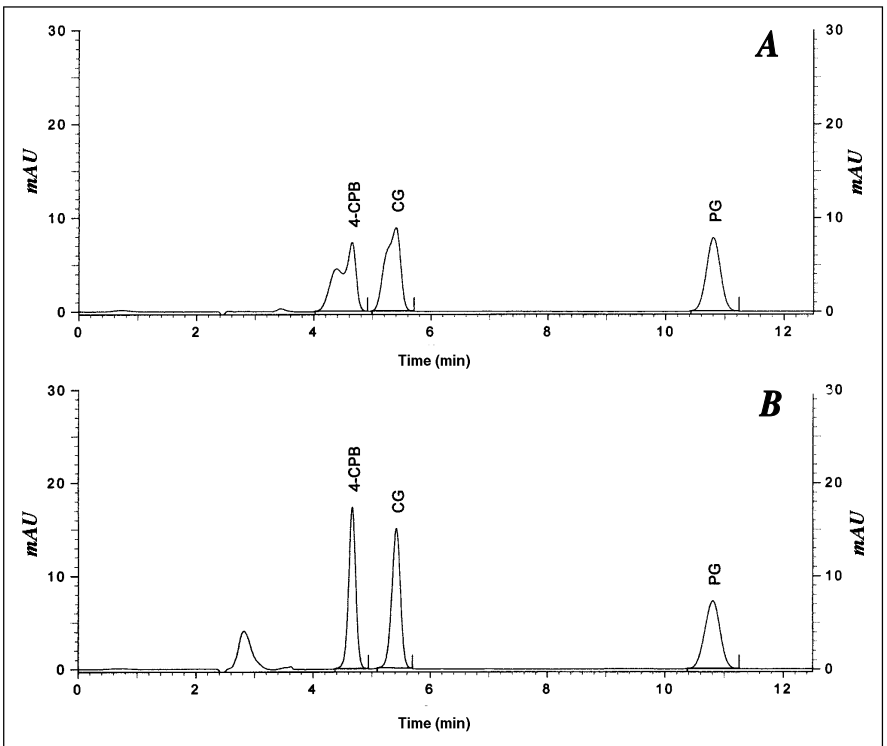

Figure 4. Chromatograms of the basic analytes dissolved in 50% ACN (A) versus 90% methanol (B).

Dilution with buffer after precipitation was necessary to obtain correct pH and to prevent high losses of the basic substances in the loading step. Experiments with only 10% ACN in the loading step showed a high nonreproducible breakthrough of ATQ. This can be explained by limited solubility and shows that it is necessary with complete solubilization in the loading step for efficient and reproducible extraction to the binding sites. The basic substances had significantly higher recoveries (~ 85%) when the loading and washing step was limited to 10% ACN. The loss of the basic substances at the chosen loading and washing step was however reproducible, though the loss of ATQ caused by limited solubility at low ACN levels was not. Gaillard et al. reported an SPE method for simultaneous determination of HFA together with other basic, acidic, and neutral analytes from hair. HFA was extracted from hair using acidified water (30). The recovery of HFA was only 65%, but it was stated that it could be improved to

approximately 86% with reference to earlier work (30,42). The earlier method involved protein precipitation combined with SPE and used a minimum of 50% ACN throughout the method. It is most likely that limited solubility for HFA in acidified water was the main reason for the lower recovery and higher RSD with the new method.

No difference in analyte response was observed using the vials and inserts of different material. A significant adsorption of the basic substances to glass tubes was observed when the processed plasma eluates were evaporated. The responses for the basic analytes were 30–50% lower when evaporated in glass tubes compared with evaporation in plastic tubes. ATQ and ATQ–IS were slightly lower when evaporated in glass tubes compared with plastic tubes. This experiment clearly shows that adsorption is highly dependent upon the circumstances. Adsorption to material for analytes when in one specific solution does not necessarily reflect adsorption characteristics during every step in the method.

Assay validation

Table II shows a summary of the precision and accuracy during the validation. At the LOQ 25nM for the bases and 50nM for ATQ, the s/n was greater than 10:1, and the intra-assay precision was approximately 15%. At the LOD 15nM for the bases and 20nM for ATQ, the s/n was greater than 3:1. Figure 6 shows a chromatogram for the lowest precision sample (97nM).

Linear calibration curves were obtained with correlation coefficients of 0.998 or better for all analytes. The calibration curves were obtained during 6 days. It was necessary to divide the calibration range into two calibration curves for ATQ because the highest calibration point was 300 times the lowest calibration point. One calibration curve was made for the standards in the range 50–1000mM and another one for all the standards.

The recoveries of ATQ and ATQ–IS were in the range of 90% and independent of concentration. The recoveries of the basic analytes and their internal standards were significantly lower (i.e., ~ 60%) because of some loss in the SPE loading and wash step. Experiments in our laboratory showed that interactions with weak ion exchangers are affected by the amount of organic modifier in the sample while interactions with strong ion exchanger are more or less independent of the amount organic modifier in the sample. The precision data suggests, however, that this loss was constant and reproducible.

The only analytes that were affected by the different times in the heating block were CG and CG–IS, which were reduced

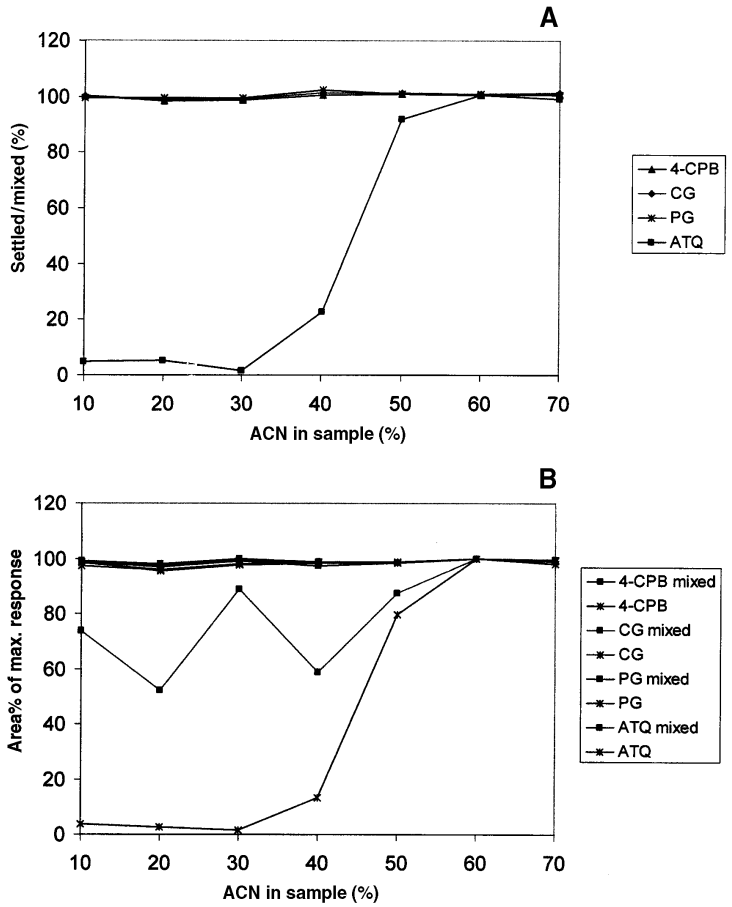

Figure 5. Solubility for ATQ, PG, CG, and 4-CPB with different ACN content in the sample. Ratio between samples settled for 24 h and mixed prior to LC injection (A) and percentage response relative to maximum response for individual samples (B).

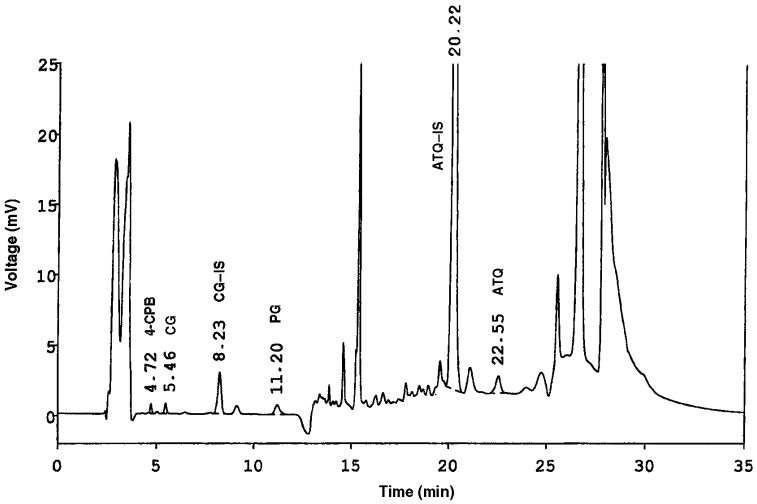

Figure 6. Chromatogram for a spiked plasma sample (97nM).

with approximatley 90% when stored the additional hour at 100°C. All analytes were stable when stored until dried and for an additional 60 min. The result indicated that CG and the analogue CG–IS are heat sensitive and that evaporation should be carried out at the lower temperature of 60°C to avoid degradation.

No endogenous peaks that would interfere with the quantitation of the analytes were observed from extracted blank plasma. The selectivity for several antimalarials was investigated for the same LC method, and none of them showed any interference effect for the quantitation of the analytes.

Conclusion

A method for the simultaneous determination of highly lipophilic and strong basic hydrophilic analytes has been developed and validated. It has been shown that it is essential with an extensive evaluation of adsorption and solubility when analytes with different properties are to be simultaneously determined. The method has been developed as a compromise between analyte recovery and reproducible quantitation. The new mixed-mode column will hopefully be a successful complement to the existing mixed modes within toxicological screening assays and, in particular, for quaternary amines and polybasic analytes.

Table II. Accuracy and Within- and Between-Day Precision of ATQ and Bases Added to Plasma

		Added (nmol/L)	Mean	RSD (%)	%Deviation (found vs. added)
Within-day	PG	96	91.2	4.8	–5.0
(*n* = 18)		903	938.8	3.4	4.0
		1790	1824.3	3.2	1.9
	CG	96	95.7	2.8	–0.3
		903	925.2	2.6	2.5
		1790	1810.1	3.4	1.1
	4-CPB	96	93.8	2.9	–2.3
		903	925.6	2.7	2.5
		1790	1797.7	3.1	0.4
	ATQ	97	111.3	2.2	14.7
		870	870.1	2.6	0.0
		11850	11782.5	3.3	–0.6
Between-day	PG	96	91.2	8.9	
(*n* = 6)		903	938.8	2.2	
		1790	1824.3	1.8	
	CG	96	95.7	6.0	
		903	925.2	2.7	
		1790	1810.1	1.2	
	4-CPB	96	93.8	6.7	
		903	925.6	3.5	
		1790	1797.7	1.2	
	ATQ	97	111.3	12.2	
		870	870.1	1.7	
		11850	11782.5	4.1	

Acknowledgments

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