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ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 727–732
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Assay of artemether, methylparaben and propylparaben in a formulated paediatric antimalarial dry suspension

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Received 12 May 2006; received in revised form 12 July 2006; accepted 14 July 2006
Available online 30 October 2006

Abstract

Two HPLC–UV methods are described for the separate determination of artemether (AM) and the combined preservatives, methylparaben and propylparaben in a pharmaceutical dosage form. These analytes are contained in a dry suspension with a high amount of non-soluble excipients, some of which can interfere with the analysis. This makes their separation and analysis of the actives complex. Moreover, due to the wide difference in concentrations, the three analytes could not be quantitated simultaneously. Artemether was analysed using a reversed-phase Nucleosil® C_{18} column [5 μm, 125 mm × 4 mm (i.d.)] with a mixture of acetonitrile: potassium phosphate buffer pH 5.0 (0.05 M): water [48:32:10 (v/v/v)] as mobile phase. Due to the low solubility of the hydroxy benzoic acid esters in water, their sodium salts were used in the formulation. Complete separation of these preservatives was achieved on the same type of column as artemether using as eluent acetonitrile: potassium phosphate buffer pH 5.0 (0.05 M) (30:70, v/v). Quantitation was achieved with UV detection at 215 nm for artemether and 254 nm for the parabens, respectively. And in both methods, pump flow rate was 1.0 ml/min, sample injection volume 20 μl, ambient temperature maintained and no prior sample extraction methods were necessary throughout the experiments. Calibration curves were linear at concentration ranges of 4–16 μg/ml, 1–4 μg/ml and 1–10 mg/ml for methylparaben, propylparaben and artemether respectively. The excipient powder interference could be eliminated by diluting the sample and the analytes eluted at relatively short times using these systems. Both methods were further validated in terms of specificity, linearity, precision and accuracy. The procedures prescribed here are simple, selective and can be used for routine quality control and stability indicating tests involving the analysed compounds formulated in complex matrices.
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Keywords: Active ingredient; Preservatives; Artemether; Reversed-phase HPLC; Parabens; Method validation

1. Introduction

Malaria is an established public health problem in several third world countries. There are 300–500 million clinical cases each year, and between one and three million deaths, mostly children (and pregnant women) are attributable to this disease [1]. These estimates render malaria the pre-eminent tropical disease and one of the top three killers among communicable diseases [2]. The arrival of resistance against standard drugs like chloroquine and sulphadoxine–pyrimethamine have worsened the situation, therefore the challenge ahead lies in determining the best alternative therapies available for use now.

Artemisinin-like compounds have been used successfully in the recent years to treat malaria. These compounds are very effective against chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* [3].

In earlier work a paediatric dry suspension had been developed with artemether as active compound [4]. The concentration of this active is 180 mg/60 ml after reconstitution in water. A dry powder product with these characteristics offers several advantages: maintenance of the chemical stability of the drug until (and after) reconstitution, reduction in transportation cost and possibility to adapt the dosage for different age groups. Once the particles have been wetted, they must be distributed uniformly throughout the liquid medium. The preparation contains macromolecules as suspending agents with thixotropic behaviour. Other solid excipients include taste and colouring agents. In addition, a fairly high amount of Aerosil® 200 was added to protect the active ingredient against moisture. Hence, this makes the preparation to contain a high amount of solid excipient powder making the analysis of the active ingredient and preservatives complex.

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doi:10.1016/j.jpba.2006.07.053

The presence of a suitable preservative in such a preparation is required since the powder has to be reconstituted in water. Several classes of these antimicrobial agents exist and for oral formulations the choice is limited to sorbic acid, benzoic acid, and the parabens. Moreover, we have to be more cautious in the preservative choice since the product is foreseen for small children less than 5 years old. Therefore, a combination of sodium methylparahydroxybenzoate or methylparaben (MP) and sodium propylparahydroxybenzoate or propylparaben (PP) were chosen for their broad antimicrobial spectrum, pH stability and safety [5,6]. It is advisable to keep the total level of the combined preservatives to the minimum due to their tendencies to induce allergic reactions [7].

There are numerous published assays for the quantification of artemether (AM) alone or simultaneously with its metabolite (dihydroartemisinin). However, most of these assay use HPLC systems which require prior extensive method development, e.g. electrochemical detection [8,9] and a higher degree of operator expertise and maintenance, e.g. HPLC–mass spectrometry [10] than that required by UV detectors.

Several studies have also described the analysis of artemisinin derivatives in a complex matrix such as plasma [11–13].

In the tropical countries where the disease burden is high, it is important to develop analytical techniques that are simple and fast with a lesser potential for equipment problems. For that reason, a simple HPLC–UV analysis was tried.

Methods have also been developed for the analysis of parabens in oral soluble liquid formulations like syrups and eye drops [14–17]. But none has successfully applied this to the analysis of the actives in dry powder mixtures with high insoluble ingredients and nothing is published on the analysis of artemether in the presence of preservatives.

In this work different HPLC methods with ultraviolet detection (isocratic and gradient) were tested for the simultaneous (if possible) analysis of artemether and the parabens in the formulation. The methods described here have been developed and validated to separate the determination of artemether and the parabens. AM was analysed using a one-point calibration method, while a standard addition method and a one-point calibration method were tested for the two parabens, from which the latter was selected for further sample analysis and validation.

2. Experimental

2.1. Materials and reagents

Potassium dihydrogen phosphate and sodium hydroxide crystals (both Ph. Eur grade) were obtained from Merck (Darmstadt, Germany) and HPLC grade methanol and acetonitrile were supplied by Fisher Scientific (Leicestershire, UK). Sodium methylparaben was bought from Federa (CERTA NV, Belgium) and sodium propylparaben was obtained from Clariant (Sulzbach, Germany). Artemether was kindly provided by Arenco Pharmaceutica (Geel, Belgium). In all experiments ultra pure water from Milli-Q, Millipore Corporation (Bedford, MA, USA) was used. All other reagents were of analytical grade.

2.2. Chromatographic apparatus and conditions

Two different chromatographic systems were experimented: gradient elution (for simultaneous analysis of AM, MP and PP respectively) and isocratic separation. In the isocratic mode, artemether was analysed using a Merck-Hitachi L-6000 pump, a Perkin-Elmer LC 90 UV spectrophotometric detector and a Merck-Hitachi, D-2500 Chromato-Integrator. The column was a Nucleosil, 120-5 C_{18} [125 mm × 4 m (i.d.), 5 μm particle size] from Macherey-Nagel (Düren, Germany). A degassed mixture of acetonitrile: potassium dihydrogen phosphate solution 0.05 M, pH 5.0: water (48:32:10, v/v/v) was used as eluent. Quantitation of AM was performed at 215 nm.

The chromatographic apparatus for the parabens was made up of a Merck-Hitachi LaChrom L-7100 pump (loop 20 μl), a Merck-Hitachi L-7400 UV detector and a Merck-Hitachi D-7500 Integrator, all from Hitachi Ltd. (Tokyo, Japan). The stationary phase was a Nucleosil C_{18} [125 mm x 4 mm (i.d.)] column from Macherey-Nagel (Düren, Germany) and detection was at 254 nm. Various conditions such as altering mobile phase composition, changing analyte concentration and detection wavelength were tested to obtain the optimal selective conditions for the separation of the compounds of interest. The final mobile phase for the validation of the preservatives consisted of acetonitrile: potassium dihydrogen phosphate buffer 0.05 M, pH 5.0 (30:70, v/v). The buffer was adjusted to the required pH with NaOH and filtered using a 0.45 μm diameter pore-size filter. The pumps were put under isocratic conditions at a constant flow rate of 1.0 ml/min and all separations carried out at ambient temperature.

For the gradient separation, the mobile phase composition was as follows: acetonitrile: potassium dihydrogen phosphate buffer 0.05 M, pH 5.0 (70:30, v/v) from 0 to 1 min and acetonitrile: potassium dihydrogen phosphate buffer 0.05 M, pH 5.0 (65:35, v/v), from 1 to 23 min. A re-equilibration time of 5 min was employed between injections and the pump maintained at a flow of 1.0 ml/min and detection was at 215 nm. The HPLC apparatus and column was the same as that used for the analysis of the parabens.

2.3. pH of reconstituted suspensions

The pH of the reconstituted suspensions was also checked to determine the amount of citric acid monohydrate to employ in the powder.

3. Methods of analysis

3.1. Calibration curves

Separate calibration curves were constructed for artemether and the parabens. A stock solution containing 249.8 mg of artemether was dissolved in a 25-ml flask with methanol and serial dilutions were constituted in methanol to make concentrations between 1 and 10 mg/ml.

For the parabens, the suspension was formulated using the sodium salts of the parabens. First a mixture of sodium MP,

sodium PP and CA was prepared. From this a suitable stock solution was prepared by dissolving a known powder amount in a mixture of methanol: water (80:20, v/v). Aliquots of five concentration levels were taken and diluted with pure methanol to obtain final concentrations in the range of 4–16 μg/ml for MP and 1–4 μg/ml for PP respectively. A calibration line was constructed and the linear relationship was evaluated by the method of least square analysis from the regression line.

3.2. Standard addition method for the determination of preservatives

To eliminate the influence of the matrix and moisture content (if any) on the powder mixture, a standard addition method was first tested as recommended by the ICH [18].

In this method, a stock solution containing the sodium salts of the parabens (together with citric acid monohydrate) with a concentration about three times higher than the 100% level was dissolved in a 50-ml flask using a methanol: water (80:20, v/v) solvent mixture. This was the spiking solution. The formulated suspension powder comprising all ingredients was then shared in different flasks which served as sample and standard solutions. To the latter flasks, the spiking solution was added in increasing amounts. Methanol: water solvent mixture was added and the whole mixed, ultrasonicated and centrifuged at 3000 rpm ($g = 1512$) for 15 min. The supernatant was diluted 25× with methanol for HPLC analysis. Six separate samples were prepared for the 100% level.

3.3. One-point calibration method

A bulk dry powder mixture was prepared in the absence of both artemether and preservatives (blank). An amount containing the dose (60-ml suspension) was weighed and weighed analytes were added to the blank powder in order to avoid loss, e.g. during bulk powder mixing. The whole was thoroughly mixed on a Turbula® mixer (Schatz, Basel, Switzerland) for at least 5 min.

To this, exactly 50 ml of a solvent mixture of methanol: water (80:20, v/v) was added, since the powder volume alone has a significant influence on the recovery. The flasks were then vortexed, shaken for 1 h, ultrasonicated and centrifuged at 3000 rpm ($g = 1512$) for 15 min. For AM analysis, the supernatant was analysed undiluted while the parabens were diluted appropriately before analysis. In both analyses, their reference solutions were prepared following the same procedure as the samples.

Different samples were prepared each for the 80%, 100% and 120% levels. For each, three standard solutions were prepared and used to validate the analytes for linearity, accuracy, precision and specificity.

4. Statistical analysis

The Student *t*-test was performed to validate the accuracy of the different concentration levels at the 95% confidence limit while the %R.S.D. compared the precision. The linearity of analyte concentration was checked by the correlation coefficient (r) while spot spreading around the line was evaluated by calculating the quality coefficient (QC) for the standard addition method.

5. Results and discussion

5.1. pH evaluation

pH verification was first evaluated because this factor significantly influences the degradation of artemether and the efficacy of the preservatives. During the formulation process, ±100% artemether was found to be stable within a pH range of 4–7. On the other hand, parabens act best at a pH range of 4–7 (pK_a 8.4) in the undissociated form, because at higher pH the phenol group ionizes and their antimicrobial efficiency is diminished. Thus, a final pH of approximately 6.50 was suitable for the stability of both the preservatives and artemether. The mean pH of the blank powder was 6.20; after adding sodium MP and sodium PP it increased to 8.5 and after adjusting with citric acid monohydrate, the mean pH for the 80%, 100% and 120% levels fell respectively to 6.60, 6.53 and 6.50.

6. HPLC development method

6.1. Optimisation of the chromatographic conditions

During sample preparation different ratios of methanol: water mixture were tested to check the dissolution of the three analytes in the same flask. A ratio of 80:20 (v/v) methanol: water completely dissolved all three without precipitation. Here, the water phase is required to dissolve the sodium salts of the parabens and citric acid monohydrate which then react to form the respective MP and PP. These products thereafter dissolve in the methanol phase and can be analysed. Parabens possess UV absorbable chromophores with a high extinction coefficient while artemether lacks this moiety. Therefore, simultaneous analysis must be executed at 215 nm. Initial attempts were made to develop a system that would (in future) be suitable for the analysis of both artemether and the preservatives simultaneously within a reasonable time on the same apparatus with detection at 215 nm. The advantage here will be a reduction in the sample preparation steps in addition to using a one system apparatus. Due to the variation in the physico-chemical properties of artemether and the parabens, ion-pair chromatography could be a good method to analyse all three analytes. To do this a modifier is needed to increase the pH of the medium past the pK_a of the parabens. This is necessary to ionise the species thereby altering their retention. This method was not feasible with respect to this system due to the high pK_a of the parabens and the silica supported on bonded columns is only stable within a narrow pH range (2–8). Therefore, gradient elution was tried.

There was a constant interfering peak that disturbed the resolution of PP. Several attempts to separate these peaks were unsuccessful. However, further sample dilution eliminated the unwanted peak and this system was capable of separating the

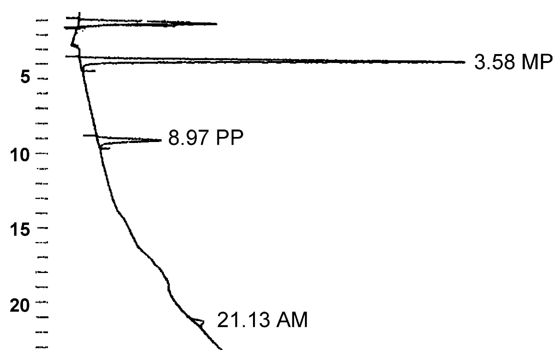

Fig. 1. Chromatogram showing the separation of standard solutions of 0.48 mg methylparaben (MP) and 0.12 mg propylparaben (PP), 1.8 mg artemether (AM) per 50 ml solvent (elution in gradient mode) and detected at 215 nm.

three compounds with good resolution with the following retention times recorded: 3.58, 8.97 and 21.13 min for methylparaben, propylparaben and artemether respectively, but at the same time reduced the concentration of artemether beyond quantification (Fig. 1). Due to this hindrance, gradient elution was discarded as the three products could not be adequately analysed in a single run.

In the isocratic mode, artemether was well separated from the powder matrix peak using an acetonitrile: potassium dihydrogen phosphate buffer (0.05 M): water (48:32:10, v/v/v) mixture as mobile phase with a retention time of ca. 9 min. Using this system MP eluted with the solvent front and reducing the organic phase volume in the mobile phase retarded AM elution; therefore different mobile phase compositions were adopted for both analyses.

MP and PP were initially separated at 254 nm on a C_{18} column using acetonitrile: potassium hydrogen phosphate buffer (0.05 M), pH 5.0: water (22.5:49:28.5, v/v/v) mixture and MP eluted after 5 min while PP eluted as late as 28 min. Substituting the buffer with acetic acid (5%) and changing the acetonitrile concentration to 35% and that of water to 65% gave retention times of 2 min and 7 min for MP and PP respectively, however with an interference peak at 5 min. Morever, the capacity factor of the first analyte was too low. The final mobile phase of ACN: KH_2PO_4 buffer 0.05 M, pH. 5.0 (30:70, v/v) for the parabens were chosen due to its efficiency in separating the two parabens. MP eluted approximately at 3.57 min while the retention time of PP was 11.52 min (Fig. 2).

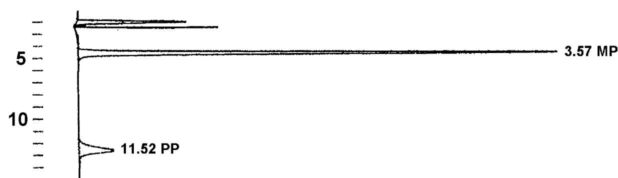

Fig. 2. Isocratic separation of diluted dry suspension spiked with 0.48 mg and 0.12 mg per 50 ml solvent of methylparaben (MP) and propylparaben (PP) respectively. Experimental conditions: phosphate buffer (0.05 M), pH 5.0; Acetonitrile (70/30 v/v), UV detection was at 254 nm and flow rate = 1.0 ml/min.

7. Standard addition

With this method, the effect of the excipients and water content on the recovery of the analytes in the samples is eliminated. A standard curve was constructed with sample concentration as abscissa and peak response as ordinate.

From this the theoritical concentration of the sample (C_o), mg/50 ml was calculated using the formula:

$$C_o = \frac{b}{a} \times \frac{V_e}{V_o} \tag{1}$$

where b = intercept on y-axis, a = slope, V_e = end volume and V_o = original volume of sample.

Only the 100% level was tested. 99.40% recovery ($t_{\alpha,(n-1)} = 0.315$) was obtained for methylparaben and 97.72% ($t_{\alpha,(n-1)} = 0.846$) was obtained for propylparaben for the 100% level with a %R.S.D. of 4.65 for MP and 6.70 for PP respectively. Though the recoveries are fairly good this method may not be suitable for stability studies due to its high and varying %R.S.D. (Table 1). Although good linearity was obtained for the six curves with the correlation coefficients of 1.00, this method may not be very precise since the recovery is extrapolated from both the slope and intercept of the line (see equation). Hence, a slight change in any (or both) parameters dramatically affects the recovery data (Table 1).

8. One-point calibration method

8.1. Linearity

This is a straight line relationship between the instrumental response and the concentration of the calibration samples. Different concentration levels of standard solution were prepared and injected on the chromatograph. Linearity was determined by representing the variation peak areas against standard concentrations (μg/ml). Good linearity was found for methylparaben within the range 4–16 μg/ml (r = 1.00), 1–4 μg/ml (r = 1.00) for propylparaben and 1–10 mg/ml (r = 1.00) for artemether respectively.

8.2. Precision

Precision is defined as the closeness or agreement between independent test results, obtained under specific conditions. Two precision steps were determined.

8.3. Injection (intra-day) precision

This was done by injecting the same sample six times on the chromatograph on the same day with the same analyst using the same mobile phase and the %R.S.D. was used to determine the injection precision. The %R.S.D. were respectively 0.23%, 0.17% and 0.64% (limit 1%) for AM, MP and PP (Table 2).

Table 1
Analytical data of the standard addition method for the determination of parabens (100% level) by HPLC

Parabens	mg/50 ml added	%Found	Slope	Intercept	r	%QC (limit = 2.5)
MP	48.48	105.07	1836747	784746	1.00	0.32
	48.03	98.15	1866661	916391	1.00	0.67
	47.98	105.30	1807581	946304	1.00	0.55
	47.93	96.48	1875612	903336	1.00	0.37
	48.13	94.34	1933520	912213	1.00	0.23
	48.18	97.07	1841241	898855	1.00	0.07
Mean	48.12	99.40	1860227	893641		
S.D.	0.199	4.65				
%R.S.D.	0.413	4.68				
PP	12.13	107.33	1440242	153892	1.00	0.89
	12.01	93.79	1491654	171553	1.00	0.38
	11.99	102.70	1422874	178199	1.00	0.84
	11.99	89.61	1510452	165619	1.00	0.62
	12.04	99.29	1415330	172353	1.00	0.02
	12.05	93.60	1475282	170271	1.00	0.19
Mean	12.04	97.72	1459306	168648		
S.D.	0.053	6.60				
%R.S.D.	0.439	6.75				

See text for chromatograpic conditions (QC, quality coefficient; r, correlation coefficient).

Table 2
HPLC linearity, specificity and precision of artemether, methyl and propylparaben analyses

Analyte	Linearity range (μg/ml)	r	k	System precision %R.S.D.	Method precision %R.S.D. ($n=18$)		
					80%	100%	120%
AM	10^3–10^4	1.00	2.01	0.23	0.30	1.50	0.20
MP	4–16	1.00	2.80	0.17	2.00	1.84	2.16
PP	1–4	1.00	11.45	0.64	2.78	2.95	1.38

k, retention (capacity) factor; r, correlation coefficient; R.S.D., relative standard deviation.

8.4. Method (inter-day) precision

This was calculated as repeated injections of multiple sample preparations ($n=6$ each at the 80%, 100% and 120% concentration levels) for AM, MP and PP. Three injections of each sample were done. The average retention times for each analyte were checked and the %R.S.D. of the peak areas was always lower than 3% (limit 5%) for the three analytes (Table 2).

8.5. Accuracy

This was performed to check the recovery of the analytes. Known amounts of artemether and preservatives were added

Table 3
Accuracy assessment of one-point calibration method for AM, MP and PP assays [S.D. = standard deviation, $t_{theor\cdot(\alpha,n-1)} = 2.57$ ($n=6$), 4.30 ($n=3$), 2.23 ($n=12$) and 2.11 ($n=18$)]

	mg/50 ml added (mean)	% Recovered (mean)	$t_{calc.(\alpha,n-1)}$	S.D.
AM				
80%	150	100.30	1.85	0.28 ($n=3$)
100%	183	100.50	0.79	1.55 ($n=6$)
120%	220	101.27	10.47	0.21 ($n=3$)
Mean (3 levels)		100.69	2.13	1.12 ($n=12$)
MP				
80%	38.5	97.33	3.37	1.94 ($n=6$)
100%	48.0	101.57	2.10	1.84 ($n=6$)
120%	57.6	100.21	0.24	2.17 ($n=6$)
Mean (3 levels)		99.70	0.64	1.98 ($n=18$)
PP				
80%	9.6	102.11	1.82	2.84 ($n=6$)
100%	12.0	101.88	1.54	3.00 ($n=6$)
120%	14.4	99.07	0.95	1.37 ($n=6$)
Mean (3 levels)		101.02	1.80	2.40 ($n=18$)

to the blank suspension and their recovery efficiency estimated as the percentage found compared to the actual amount added. Three standard solutions for each level were prepared and their theoritical recovery calculated against these standards. The results are presented in Table 3. The mean measured concentration was found to be 100.69% for AM, 99.70% and 101.02% for methylparaben and propylparaben respectively for the three levels. A Student *t*-test was used to statistically check the above accuracy at the 95% significance level. All calculated *t*-values were less than *t*-theoritical except for the 120% artemether level and 80% MP level respectively. However, taken the three levels together, the overall accuracy conformed. This proves that both methods can be used with certainty in the analysis of the three products.

9. Conclusions

In this study, the separation of three pharmaceutical compounds using two chromatographic systems has been presented. The concentrations of the analytes differ greatly therefore, gradient elution could not be used to simultaneously quantitate the three analytes. Furthermore, ion-pair chromatography was not possible. As shown, the analysis of the parabens using the standard addition method is time consuming and of little value for stability indicating studies due to the wide variation in its recovery data. However, this method is precise for the determination of the content of active and preservatives in the suspension. On the other hand, it was shown that the one-point calibration method is a simple, fast and generates better reproducibility and accurate results. By adding an exact volume, the influence of powder volume which was found to be ca. 4.5 ml (data not shown) on 50 ml is eliminated. On the basis of the chromatographic conditions of the compounds, optimum conditions with good peak symmetry, distinct separation among the analytes and excipients was found.

The short retention times indicates that several analyses can be performed within a short time frame. In addition, the ability of the system to analyse two parabens simultaneously reflects the potential and suitability of the method as two parabens usually co-exist in the same formulation.

The validation data exhibited by the linearity, precision and selectivity convincingly demonstrates that these methods will also permit the analysis of dry suspensions already present on the market.

Acknowledgement

The authors would like to sincerely thank Katelijne De Cock for the excellent technical assistance in the experiments.

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