ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1996) 419–422

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Short communication

Validation of the assay method for camphor and menthol in a herbal drug preparation[1]

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Received for review 4 April 1996

Keywords: Validation; Gas chromatography; Camphor; Menthol; Herbal drug preparation

1. Introduction

Routine quality control of a herbal drug preparation requires the identification and determination of the contents of active ingredients in the product [1,2]. Measurement alone is not sufficient; it is moreover necessary to know how objectively to evaluate the experimental results [3–5]. This includes validation of the method for quantitative determination of the ingredients in a herbal drug preparation [6,7].

The contents of active ingredients, camphor and menthol, in a commercial herbal drug preparation were determined by gas chromatography. The aim of the work was to examine the validity of this method for the determination of the contents of the ingredients. Before the assessment, risk analysis was carried out in order to direct the validation to the characteristics with the greatest error risk, namely solution stability, linearity response of the standard solutions, accuracy, precision and ruggedness of the method

2. Experimental

2.1. Apparatus

A Perkin-Elmer Model 3920B gas chromatograph was used with a flame-ionization detector connected to an M-2 integrator (Perkin-Elmer, Norwalk, CT).

2.2. Chemicals

Methanol (reagent grade) was obtained from Kemika (Zagreb, Croatia). The purities of (−) fenchone (100%) from Carl Roth (Karlsruhe, Germany), camphor (96.67%) from Irex-Aroma (Zagreb, Croatia) and menthol (98.45%) from Boulgarel (Marseille, France) were determined by the GLC method of peak normalization.

A commercial herbal drug preparation of nominal composition camphor 2.70% and menthol 2.00% and a placebo of the herbal drug prepara-

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[1] Presented at the Fifth International Symposium on Drug Analysis, September 1995, Leuven, Belgium.

PII S0731-7085(96)01850-X

Table 1
Response factor of reference solution during 60 days[a]

Camphor (%)					Menthol (%)				
0 h	24 h	48 h	30 days	60 days	0 h	24 h	48 h	30 days	60 days
1.0351	1.0848	1.0834	1.0447[b]	1.0088[b]	1.0456	1.0920	1.0671	1.0679	1.0107[b]
1.0652	1.0652	1.0685	1.0429[b]	1.0388[b]	1.0632	1.1065	1.0507	1.0447	0.9610[b]
1.0687	1.0757	1.0703	1.0326[b]	1.0101[b]	1.0382	1.1259	1.0743	1.0495	0.9846[b]
1.0864	1.0767	1.0568	1.0560[b]	1.0147[b]	1.1442	1.1165	1.1243	1.0285	0.9583[b]
1.1076	1.0645	1.0641	1.0530[b]	1.0160[b]	1.0944	1.0874	1.0518	1.0260	0.9678[b]

[a]Five replicate injections of the same solution.
[b]Significant at $p < 0.05$.

tion were prepared in the laboratories of Saponia (Osijek, Croatia).

2.3. Test procedure

2.3.1. Standard solutions

Internal standard solution: (−)-fenchone, 20 mg ml^{-1} (±0.0001 g) in methanol. Standard solution: amounts of 300 mg (±0.0001 g) of camphor and 100 mg (±0.0001 g) of menthol were dissolved in methanol in a 100 ml calibrated flask and diluted to volume. Reference solution: 1 ml of standard solution and 1 ml of internal standard solution were mixed by shaking in a test-tube fitted with a ground-glass stopper.

2.3.2. Sample solutions

Solution of the herbal drug preparation: 0.7 g (±0.0001 g) of the herbal drug preparation was weighed into a test-tube fitted with a ground-glass stopper and 1 ml of methanol and 1 ml of internal standard solution were added. Solution of the placebo: 0.7 g (±0.0001 g) of placebo was weighed into a test-tube fitted with a ground-glass stopper and 1 ml of methanol was added. The herbal drug preparation and the placebo solutions were shaken well after preparation and left to stand for 30 min. The samples for chromatography were taken from the upper, clear methanolic layer.

2.3.3. Measurements

GLC measurements were performed on a 2 m × 1.75 mm i.d. glass column packed with Carbowax 20M on Chromosorb W HP (100–120 mesh). The temperature programme was initially 75°C held for 5 min, then increased at 4°C min^{-1} to 200°C, which was held for 15 min. The gas flow rates were carrier gas (nitrogen) 30, hydrogen 20 and air 18 ml min^{-1}. Data were processed by the method of peak normalization (data obtained from purity testing and chromatography of the internal standard solution, standard solution and reference solution) and the internal standard method (data obtained from chromatography of the herbal drug preparation and spiked placebo).

2.4. Validation of the test procedure

2.4.1. Solution stability

The camphor and menthol contents (%) were compared in the standard solution and reference solution immediately after the solutions had been prepared and after storage in a refrigerator for 24 h, 48 h, 30 days and 60 days. In the solution of the herbal drug preparation, camphor and menthol were determined immediately after the solution had been prepared, then after 24 and 48 h. The fenchone content was examined in the internal standard solution immediately after preparation and after 1, 2, 30 and 60 days.

2.4.2. Linearity

The camphor and methanol contents were determined in standard solutions prepared in the range 50–150% of the nominal camphor and menthol concentrations. Each solution was injected five times

Table 2
Camphor and menthol contents in the placebo solution injected with camphor and menthol solution of known concentration[a]

Aliquot	Camphor				Menthol			
	Added (%)	Found (%)	Recovery (%)	Relative error (%)	Added (%)	Found (%)	Recovery (%)	Relative error (%)
1	2.17	2.16	99.54	−0.46	1.36	1.39	102.21	+2.21
	2.20	2.12	96.36	−3.64	1.37	1.32	96.35	−3.65
	2.17	1.96	90.32	−9.68	1.36	1.32	97.05	−2.95
2	2.43	2.33	95.88	−4.12	1.62	1.58	97.53	−2.47
	2.46	2.38	96.75	−3.25	1.66	1.67	100.61	+0.61
	2.42	2.30	93.39	−6.61	1.61	1.63	102.48	+2.48
3	2.62	2.49	95.40	−4.60	1.83	1.95	106.56	+6.56
	2.58	2.47	95.74	−4.26	1.81	1.78	97.24	−2.76
	2.62	2.54	96.95	−3.05	1.84	1.74	96.74	−3.26
4	2.89	2.84	98.27	−1.73	2.10	2.18	103.81	+3.81
	2.84	2.87	101.06	+1.06	2.06	2.11	102.43	+2.43
	2.91	2.84	97.59	−2.41	2.12	2.14	100.94	−0.94
5	3.11	3.13	100.64	+0.64	2.33	2.40	103.00	+3.00
	3.18	3.00	94.34	−5.66	2.38	2.33	97.90	−2.10
	3.16	3.15	99.68	−0.32	2.37	2.37	100.00	0.00

[a]Three independent assays at five concentrations.

2.4.3. Accuracy

The recoveries of camphor and menthol were assessed in placebo solutions with known added amounts of camphor and menthol in the range 50–150% of the nominal concentrations. The assays were carried out on three aliquots (three measurements each).

2.4.4. Precision

Five aliquots of the same sample of a herbal drug preparation were injected five times each.

2.4.5. Ruggedness

Two analysts determined camphor and menthol contents in the same sample of a herbal drug preparation in five aliquots (five measurements each).

3. Results

The results of stability tests of solutions were processed statistically using Student's t-test at $p < 0.05$. There were no significant differences in the contents of active ingredients in the internal standard solution and the standard solution after 60 days and in the herbal drug preparation and the placebo during 48 h. Significant differences in the response factor of the comparator solution were noticed 48 h after the solution had been prepared (Table 1).

The linearity of the calibration graph (peak area *versus* camphor and menthol concentrations) for both camphor and menthol standard solutions were examined. Straight lines were obtained. The regression lines, calculated by the least-squares method, were $y = 114.32 = 172357.98x$ for menthol and $y = 638.97 = 156860.01x$ for camphor, with the confidence intervals at $p = 0.05$.

The results of the assay for camphor and menthol in the placebo spiked with camphor and menthol of known concentration are shown in Table 2. The data are presented as mean values. The accuracy of the method is expressed as recovery and relative error.

The precision of the method is expressed as relative standard deviation (RSD). The results (Table 3) were processed statistically using Student's t-test.

Table 3
Camphor and menthol contents (%) in the herbal drug preparation[a]

Compound	Aliquot	Analyst I			Analyst II		
		x_i (%)	$\bar{x}$ (%)	RSD (%)	x_i (%)	$\bar{x}$ (%)	RSD (%)
Camphor	1	3.31, 3.15, 3.19, 3.18, 3.11	3.19	2.35	2.96, 2.87, 3.01, 2.95, 2.94	2.95[b]	1.71
	2	3.01, 3.09, 2.95, 2.06, 3.02	3.03	1.76	2.86, 2.89, 2.85, 2.87, 2.93	2.88[b]	1.10
	3	2.90, 3.01, 3.09, 2.88, 3.06	2.99	3.15	2.75, 2.76, 2.80, 2.82, 2.87	2.80[b]	1.73
	4	3.05, 2.91, 3.12, 2.99, 3.08	3.03	2.71	2.94, 2.88, 2.89, 2.96, 3.02	2.94[b]	1.93
	5	2.99, 2.99, 2.88, 2.86, 2.89	2.92	2.16	2.86, 3.00, 3.02, 2.85, 2.99	2.94[b]	2.79
Menthol	1	2.08, 2.10, 2.17, 2.16, 2.06	2.11	2.07	2.09, 2.03, 2.04, 2.18, 2.13	2.09	3.01
	2	2.03, 2.27, 1.99, 2.07, 1.92	2.06	6.40	2.06, 2.09, 2.04, 2.04, 2.06	2.06	1.00
	3	1.94, 2.00, 2.07, 2.03, 2.09	2.03	2.93	1.95, 1.93, 1.93, 1.95, 1.95	1.94	0.58
	4	2.01, 2.13, 2.16, 2.02, 2.14	2.09	3.41	1.98, 2.00, 2.00, 2.06, 2.06	2.02	1.85
	5	2.00, 2.07, 2.09, 2.06, 2.10	2.06	1.91	1.99, 2.08, 2.08, 1.97, 2.05	2.03	2.23

[a]Five independent assays by two analysts and five replicates for each determination.
[b]Significant at $p < 0.05$.

4. Discussion

The regression lines, obtained by checking the response linearity of the camphor and menthol standard solutions, do not pass through the origin; this implies systematic error, although the correlation coefficients for both camphor were 0.999. Hence some additional testing of linearity is needed. The cause of error can be found by the procedure suggested by Youden [8], which is based on factorial design of the experiment.

The spread of results, i.e. the RSD, was larger in the menthol assays, which is logical since the average menthol concentration in the herbal drug preparation is lower.

A change of analyst significantly ($p < 0.05$) influenced the results for camphor but had no influence in menthol assays.

5. Conclusions

The differences in the contents of active ingredients, in the solutions examined showed that the standard solution and the internal standard solution can be used within 60 days without influence on the results, whereas the reference solution and the solution of herbal drug preparation can be used within 48 h after preparation.

The standard camphor and menthol solutions gave a linear response in the range 50–150% of the nominal camphor and menthol concentrations. The intercept indicated a systematic error, which can be found by the procedure described by Youden [8].

The results for the precision and ruggedness of camphor assays indicated that this part of the method requires additional standardization and testing, whereas the results of menthol assays were satisfactory.

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