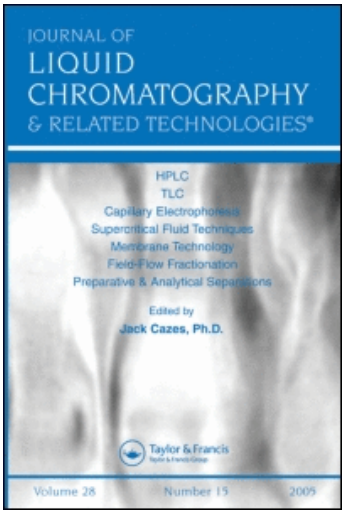


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# Optimized and validated HPLC Methods for Compendial Quality Assessment I. Methylxanthine Derivatives

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# OPTIMIZED AND VALIDATED HPLC METHODS FOR COMPENDIAL QUALITY ASSESSMENT I. METHYLXANTHINE DERIVATIVES

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## ABSTRACT

The HPLC method suggested here is suitable for the detection and quantitative determination of 0.1-1 % caffeine, theobromine and theophylline impurities in each of these methylxanthine derivatives. The system optimization includes organic modifier content, ionic strength, eluent flow rate and concludes to the use of a simple $C_{18}$/methanol-water 1:4 (I = 0.1) chromatographic system.

The limit of impurity detection was found to be 15 ng (theobromine, theophylline) and 30 ng (caffeine). The range of quantitation extends from 31.25 to 250 ng. The accuracy (percentage of recovery) and precision (repeatability) of the method are tabulated. The method, considering validation requirements of USP XXIII, demonstrates the suitability of HPLC for selective compendial purity tests.

## INTRODUCTION

The three methylxanthine (MX) alkaloids — caffeine (1,3,7-trimethylxanthine),theobromine (3,7-dimethylxanthine), theophylline (1,3-dimethylxanthine) — have reserved their therapeutic importance through several decades and they are monographed in all of the pharmacopeias, even for the present. At the purity test for "related substances" (i.e., detection of the other two MX's as impurity in the third MX derivative) the European Pharmacopeia applies thin layer chromatography; USP XXIII does not prescribe a test for related impurities. At the same time, for the RP-HPLC assay of theophylline, as internal standard, USP prescribes theobromine. In caffeine, USP XXIII applies a non-selective chemical reaction for the detection of "other alkaloids" impurity.



In our opinion, the application of HPLC for the detection (quantification) of "related impurities" (i.e., related MX impurities in the MX samples) can provide a good solution.

Although a number of works dealing with the HPLC separation of the three MX's can be found in the literature, no reference to drug purity control was found.

In a previous paper[1] dealing with purity control of MX's, the relevant literature till 1987 was compiled and tabulated.

Reviewing the papers of the period between 1988-1995, there may be found HPLC separation of the MX's in biological samples[2-12] and in foods.[13,14] The publication of Vree et al.[15] deals with the relationship of structure and chromatographic behavior of purine derivatives, and only one paper[16] was found in which MX's were separated in pharmaceutical preparations.

The present work aims to complete the cited paper[1] by chromatographic system optimization and method validation, thereby enabling the presented procedure for compendial application, including also the quantitative determinations of the detected impurities.

Even our first experiments definitely showed that the very simple RP-HPLC system $C_{18}$/methanol-water, after suitable optimization, provides a good solution of the task. A similar system was used in our previous work[1] and, by some other authors, for the separation of caffeine and theobromine in foods,[17] as well as for the three MX's in biological samples.[18]

The present paper demonstrates that the optimized system allows the resolution of the three (isomeric and homologous) MX's with a method which is also suitable for sensitive purity control. At the system optimization and method validation, the general principles of USP XXIII[19] and some other authentic sources[20,21] were considered.

## EXPERIMENTAL

### Chromatography

The HPLC apparatus comprised a Waters pump Model 510 (Millipore, USA) combined with a Rheodyne injector unit Model 7125 (Cotati, California, USA) equipped with a 10 μL loop. A Hewlett-Packard variable wavelength absorbance detector Model 1030B (220-800 nm) was used.



The equipment modules, subsequent to the pump, were thermostatted (Column Chiller, Model 7955 - Jones Chromatography, Ltd., Wales) at 25°C±0.1°. The chromatograms were recorded and the data handling was effected by a Hewlett Packard integrator Model 3396, Ser. II.

The $C_{18}$ sorbent, Hypersil 5 ODS (Shandon), particle size 5 μm, was packed into a stainless steel column (250 x 4.0 mm I.D.; BST, Budapest, Hungary).

As mobile phase, sonically degassed and filtered methanol-water mixtures with 20,40 and 60 v/v% of methanol were applied. All ready-made eluents contained sodium chloride in an amount to adjust the ionic strength to 0.075, 0.1, 0.125. The column void time was signalled by the injection of water.

Each retention data point was calculated as an average of at least three parallel runs. The eluent flow rate was adjusted to 0.6, 0.8, 1.0 mL/min. The effluent was monitored at the optimum wavelength, i.e., at 270 nm.

The columns were brought to the initial state by washing with 50 mL of methanol-water 1:4 mixture, 50 mL of 0.001 M HCl and then 100 mL of water, followed by purging with 50 mL of methanol. After each experiment, the loop was washed with 5-10 times repeated injection of 0.1 M HCl, then water and, finally, methanol.

**Table 1**

**Influence of Methanol Content on the Retention* of MX's**

| Compound | Methanol Content of Eluent, % 20 | 40 | 60 |
|---|---|---|---|
| Theobromine | 5.49 (1.9) | 3.88 (0.21) | 2.94 (≤0.1) |
| Theophylline | 8.05 (2.6) | 4.58 (0.4) | 3.24 (≤0.1) |
| Caffeine | 12.63 | 5.45 | 3.43 |

*$t_R$ in minutes.
$R_s$ values in parentheses; I (NaCl): 0.1; Eleuent flow rate: 0.8 mL/min.;
Injected: 10 μL of methanolic solution (equivalent to 125 ng MX);
Preparation: see Experimental.



## Materials

Caffeine, theobromine and theophylline samples were obtained from the manufacturers and met the requirements of the US, European, and Hungarian Pharmacopeias. The "related MX content " of these samples was controlled by HPLC and was found to be ≤0.01%. Methanol, RS HPLC grade, (Carlo Erba); water was double-distilled. Sodium chloride, 99.99% was obtained from Aldrich Chemical Co..

## Standard Solutions

The 0.1 g of MX bulk substances (see Table 1) were dissolved in 200 mL of a 1:3 mixture of 0.1 M HCl and methanol. This solution (c = 0.05%) was used, after dilution with the HCl-methanol mixture, to prepare the standard solutions.

## Test Solutions

The 1 g of the MX sample, spiked with 0.1 % of the two related MX's (see Table 4) was sonicated for 15 minutes with 10 mL of methanol and then

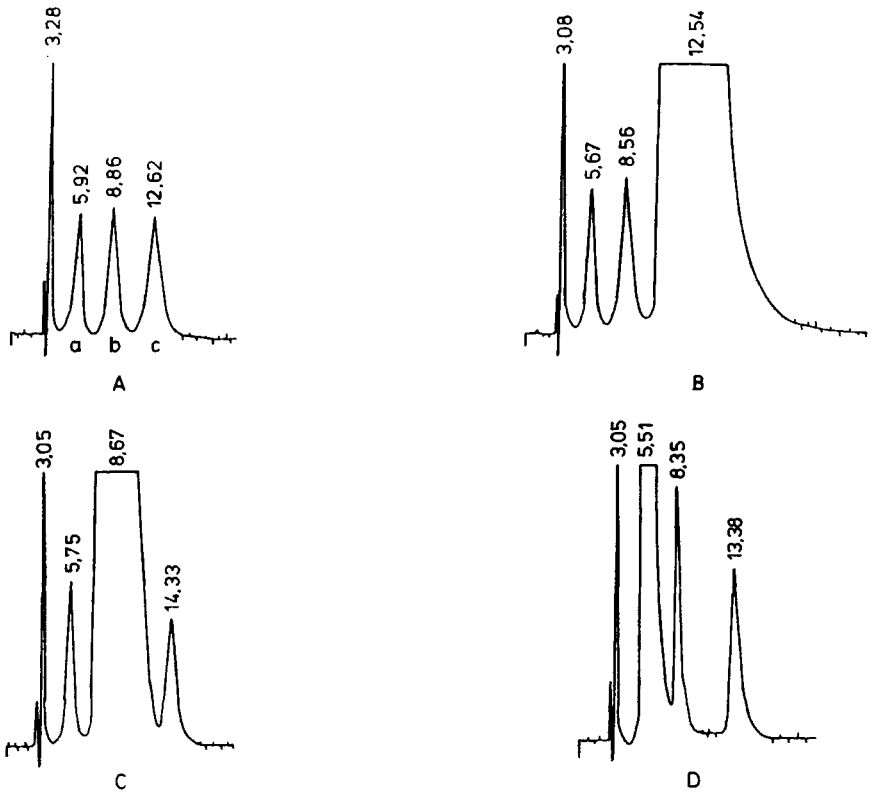




**Figure 1**. Separation of MX's and detection of related MX impurity in caffeine, theobromine and theophylline samples. **A**: 125 ng of theobromine (a) theophylline (b) and caffeine (c). **B**: 0.1% theobromine and 0.1% theophylline in caffeine. **C**: 0.1% caffeine, 0.1% theobromine in theophylline. **D**: 0.1% caffeine, 0.1% theophylline in theobromine. Injected: 10 μL of solution (125 ng of MX impurity). Preparation: see Experimental section.

filtered. To a 5 mL portion of the filtrate, was added 5 mL of 0.1M HCl and 30 mL of methanol (equivalent to 125 ng impurity/10 μL). One gram of theophylline or caffeine sample, spiked with 1% of the other two MX's, was sonicated for 25 minutes with 40 mL of methanol.

The solution, which became warm, was cooled, then filtered. To 1 mL of the filtrate, was added 4 mL of 0.1M HCl and 15 mL of methanol (equivalent to 125 ng MX impurity/10 μL).

**Table 2**

**Detection Limit for MX's**

| Compound | Detection Limit (ng) | Peak Area S.D. (%) |
|---|---|---|
| Theobromine | 8.0 | 2.8 |
| Theophylline | 8.0 | 2.8 |
| Caffeine | 30.0 | 5.5 |

Chromatographic conditions: Eluent, water/methanol 40:10; I, 0.1 (NaCl); Flow rate, 0.8 mL/min.; Detection, 270 nm.



One gram of theobromine, spiked with 1% of the other two MX's, was sonicated for 25 minutes with 20 mL of methanol. The cooled mixture was filtered. To 1 mL of the filtrate, was added 4 mL of 0.1 M HCl and 35 mL of methanol (equivalent to 125 ng MX impurity/10μL).

## RESULTS

### System Optimization

The main working parameters for the optimization of the $C_{18}$/methanol-water system were selected by applying the screening sequence which is shown in Table 1. The table shows, also, the effectiveness of the system by comparing the influence of eluent methanol content on the chromatographic behavior of MX's. In conclusion, as optimized parameters, 1:4 methanol - water content in eluent, 0.1 ionic strength (NaCl) 0.8 mL/min. eluent flow rate and 270 nm as wavelength of detection were accepted. Figure 1 illustrates that the high resolution achieved allows the sharp separation of MX's, even in the case when they are present in a ratio 1000:1:1.

### Method Validation

*Limit of detection* was determined by using the signal-to-noise ratio method. For this, the peak area of a solution with known concentration of the

**Table 3**

**Quantification Range and Linearity of Conc'n./Peak Area Relationship for MX's**

| | S.D. % (Peak Area) | | |
|---|---|---|---|
| **Injected** | **Theobromine** | **Theophylline** | **Caffeine** |
| 31.25 | 1.9 | 2.5 | --- |
| 62.50 | 3.6 | 3.9 | 2.6 |
| 93.75 | 1.9 | 1.7 | 3.4 |
| 125.0 | 2.1 | 3.1 | 2.5 |
| 187.0 | 2.3 | 1.4 | 2.3 |
| 250.0 | 2.1 | 2.1 | 1.1 |
| **r:** | 0.9996 | 0.9986 | 0.9956 |



MX, and the noise (background response of a blank experiment) were determined. The average of 6-6 runs was taken for the evaluation. As the limit of detection, a signal-to-noise ratio of 3:1 was accepted (see Table 2).

*Limit of quantitation:* (the lowest amount of MX impurity can be determined with acceptable precision and accuracy) was determined using the value of the background response.[19] The latter was obtained by analyzing eight blank experiments and calculating the standard deviation of background response. This value, multiplied by ten, gives the limit of quantitation. In this work, considering the requirements of repeatability, as limit of quantitation, 31.25 ng was chosen.

*Linearity and range:* the upper level of the range of quantitation was chosen as the highest amount of MX appearing in a peak, with acceptable peak shape and symmetry (Table 3). The linearity was characterized as the regression coefficient of test results of peak area *vs.* analyte concentration relationship (Table 3). The amount of impurity can be quantitated between 31.25 and 250 ng.

*Precision (repeatability):* was determined by dissolving 0.01 g of MX in a mixture of 150 mL methanol and 50 mL 0.1 M HCl. This solution (MX content = 0.005%) was used for the preparation of the calibration curve in the range 31.25-250 ng (Table 3). The precision was determined by assaying the MX content of five aliquots at each concentration within the above range.

**Table 4**

**Accuracy of MX-Related Impurity Determination**

| Bulk Substance | Impurity Content, % - Caffeine Calc. | Caffeine Found | Theobromine Calc. | Theobromine Found | Theophylline Calc. | Theophylline Found |
|---|---|---|---|---|---|---|
| Caffeine | --- | --- | 0.10 | 0.092 | 0.10 | 0.091 |
| | | | 1.0 | 0.90 | 1.0 | 0.91 |
| Theobromine | 0.10 | 0.088 | --- | --- | 0.10 | 0.09 |
| | 1.0 | 0.85 | | | 1.0 | 0.93 |
| Theophylline | 0.10 | 0.092 | 0.10 | 0.089 | --- | --- |
| | 1.0 | 0.91 | 1.0 | 0.089 | | |

*Accuracy* was examined by the determination "related MX" content of the samples spiked homogenously with 0.1 and 1 % of MX impurities. In conclusion, the MX's can be determined in the selected range within S.D.±2-4%. As Table 4 shows, the recovery ranges between 8-10 % in case of 0.1-1% of impurity content.



## DISCUSSION

The selected chromatographic system may be applied for compendial purity testing in two ways:

(1) performing a limit test,when the area of the related MX peak is limited to a maximum of 3 times larger then that of the noise, i.e., detection limit, (see Table 2),

(2) quantification of MX impurity content; for the evaluation a calibration curve is to be used (Table 3).

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