Simultaneous RP-IP-HPLC Assay of Theophylline, Phenobarbital, Codeine, and Ephedrine in a Suppository

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Summary. A reversed-phase ion-pairing liquid chromatographic (RP-IP-HPLC) method for simultaneous assay of theophylline (TH), phenobarbital (PB), codeine (CD), and ephedrine (EP) in an extemporaneous (magistral) suppository was developed and validated and used to investigate a reported serious adverse event. Samples were dissolved in dichloromethane and extracted by two-step liquid–liquid extraction with acetate buffer (pH 5.0) and, subsequently, 0.1 M NaOH solution. Separation was performed on an end-capped C18 silica column with stepwise gradient elution. Sample preparation and chromatographic conditions were optimized on the basis of the pK\textsubscript{a} values of the analytes. Freedom from interference from the excipients, linearity (r\textsuperscript{2} > 0.999 for all the active ingredients), range (0.01–0.08 mg mL\textsuperscript{-1} for TH, CD, and EP; 0.0025–0.02 mg mL\textsuperscript{-1} for PB), intra-day and inter-day precision, and accuracy (recovery >95% for TH, CD, and EP and >90% for PB) of the method were demonstrated. Non-compliance of the examined product was confirmed.

Key Words: RP-IP-HPLC, suppository, theophylline, phenobarbital, codeine, ephedrine

Introduction

In most European countries use of extemporaneous (magistral) pharmaceutical formulations produced by pharmacies has decreased substantially in recent decades. In special cases, however, application of such preparations is reasonable, because they enable physicians to prescribe individual treatment for their patients. Use of magistral drug formulations is especially high in paediatrics, in which appropriate dosage is often not possible using marketed drugs.

Some generally used extemporaneous preparations are included in official national formularies or in national pharmacopoeias [1–7], and others...
are ordered by individual prescriptions. For the latter, particularly, composition may vary substantially; this makes quality control difficult because of the lack of official test methods. Assay of suppositories is particularly challenging, because the excipient is usually strongly hydrophobic whereas the active components are usually quite hydrophilic.

The importance of the quality control of extemporaneous pharmaceutical preparations, however, is repeatedly confirmed by quality complaints or quality-related adverse events reported to the competent authorities by patients or hospitals. Besides being an obligation of the authority, investigation of these problems is also a major public health issue. This is explicitly stated in the requirements of good pharmacy practice (GPP) [8], where it is pointed out that “… the core of the pharmacy activity is the supply of medication and other health care products, of assured quality.”

The method presented in this study was developed to investigate the complaint of a patient about an individual extemporaneous pharmaceutical preparation. A suppository known as ‘Suppository T’ containing in 10 dosage units 100.0 mg each of theophylline (TH; drug for obstructive airway disease), ephedrine (EP; sympathomimetic) hydrochloride, and codeine (CD; cough suppressant) hydrochloride, and 25.0 mg phenobarbital (PB; antiepileptic) sodium (the chemical structures are depicted in Fig. 1.), one

![Chemical structures](image)

Fig. 1. The chemical structures of (a) theophylline (pKₐ 8.7), (b) phenobarbital sodium (pKₐ 7.4), (c) codeine (pKₐ 8.2), and (d) ephedrine (pKₐ 9.6)
Fenistil 24 retard capsule (4.00 mg dimetindene maleate per capsule; anti-histamine), and 10 g hard fat (adeps solidus; vehicle) was reported to have caused a serious adverse reaction in a 13-month-old baby. The objective of this study was to determine the amounts of the active pharmaceutical ingredients and to investigate if possible overdosage of any of these substances could be related to the reported adverse event.

Because ‘Suppository T’ is not included in the Hungarian National Formulary [1], no official assay method was available. A chromatographic assay of a tablet containing PB, EP, and TH is described in USP 32 [7]. A CE method for the same formulation has also been reported [9]. EP and CD are common ingredients of cough–cold syrups, which have been assayed by several research groups by HPLC [10–12] or capillary electrophoresis (CE) [13]. Increasing the selectivity for these two compounds during chromatographic assay in multicomponent drug compositions has also been studied [14]. Tablets containing TH and EP have been analyzed by micellar electrokinetic chromatography (MEKC) [15], RP-HPLC [16–18], and derivative spectrophotometry [18].

Although most of these methods are well-established and designed for routine analysis, none addresses simultaneous quantification of TH, PB, EP, and CD in suppositories. The RP-IP-HPLC method described in this paper is suitable for simultaneous assay of all these compounds and has been validated in accordance with the appropriate ICH guideline [19].

**Experimental**

**Chemicals, Reagents, and Solutions**

Theophylline, phenobarbital sodium, ephedrine hydrochloride, and codeine hydrochloride standard substances and hard fat (all Ph. Eur. quality) were provided by a national wholesaler (Hungaropharma, Budapest, Hungary). Fenistil 24 retard capsules were purchased from a local pharmacy.

Purified water was obtained from a Millipore Elix3 water-purifying system. Acetonitrile (ACN) and dichloromethane (CH₂Cl₂) were from Carlo Erba Reagenti (Rodano, Italy), concentrated (glacial) acetic acid (cc. CH₃COOH) from Sigma–Aldrich (Seelze, Germany), sodium acetate tri-hydrate (CH₃COONa) from Mallinckrodt Baker (Phillipsburg, NJ, USA), and sodium octane sulphonate (NaOS) from Merck (Darmstadt, Germany). NaOH (0.1 M) solution was prepared from solid NaOH from Reanal Finechemicals (Budapest, Hungary). Calibration buffers for pH measurement were from Mettler Toledo (Schwerzenbach, Switzerland).
Mobile phase component A was prepared by dissolving 1.08 g NaOS in a mixture of 20 mL cc. CH$_3$COOH, 100 mL ACN, and 900 mL water. Mobile phase component B was prepared by dissolving 1.08 g NaOS in a mixture of 20 mL cc. CH$_3$COOH, 400 mL ACN, and 600 mL water.

The acetate buffer (pH 5.0) used for sample extraction and dilution of standard solutions was prepared by dissolving 22.5 g CH$_3$COONa in 500 mL water and then adjusting the pH to 5.0 with cc. CH$_3$COOH by use of a Mettler Toledo MA 235 pH/ion analyzer.

A stock solution was prepared by dissolving TH, EP hydrochloride and CD hydrochloride (20.0 mg of each), and PB sodium (5.0 mg) in 100.0 mL of a 7:3 (v/v) mixture of acetate buffer (pH 5.0) and 0.1 M NaOH solution, resulting in a concentration level of 0.2 mg mL$^{-1}$ for TH, CD hydrochloride and EP hydrochloride, and 0.05 mg mL$^{-1}$ for PB sodium. Reference solutions were prepared by diluting 2.0 mL of the stock solution to 10.0 mL with mobile phase component A, resulting in concentrations of 0.04 mg mL$^{-1}$ for TH, EP hydrochloride, and CD hydrochloride and 0.01 mg mL$^{-1}$ for PB sodium. Working solutions for linearity testing were prepared by appropriate dilution of the stock solution with mobile phase A to furnish solutions containing 0.01, 0.02, 0.03, 0.04, 0.06, and 0.08 mg mL$^{-1}$ TH, EP hydrochloride, and CD hydrochloride and 0.0025, 0.005, 0.0075, 0.01, 0.015, and 0.02 mg mL$^{-1}$ PB sodium.

**Sample Preparation and Extraction**

Sample preparation included extraction of the compounds and subsequent dilution of the extracts. Five suppositories were gently homogenized in a mortar at room temperature. An accurately weighed quantity equivalent to approximately half of the dosage unit was dissolved in 5 mL CH$_2$Cl$_2$ and transferred to a separation funnel. The solution was shaken mechanically on an IKA KS 260 basic mechanical shaker for 5 min (150 rpm) with 3 × 5 mL acetate buffer (pH 5.0). The aqueous phases were collected in a 50 mL volumetric flask. Subsequently, the organic layer was shaken mechanically with 3 × 5 mL 0.1 M NaOH solution (10 min each, 150 rpm). The aqueous layers were added to the volumetric flask containing the acetate buffer extract and the solution was diluted to volume with the acetate buffer. Finally, 10 mL of the solution obtained was diluted to 25 mL with mobile phase component A and the solution was filtered through a Millex 0.45 μm membrane filter (Millipore Corporation, Billerica, MA, USA).
**Chromatography**

HPLC was performed with an HP 1050 LC system equipped with a diode-array detector (DAD). Results were obtained by use of HP ChemStation (A06.03) software.

Compounds were separated on a 250 mm × 4 mm PurospherStar RP-18e 5 μm column with gradient elution. Helium was used for on-line degassing. The amount of mobile phase component B was maintained at 0% from 0 to 6.5 min then increased to 35% from 6.5 to 7 min and maintained at 35% from 7 to 33 min. A 9-min post-run period was applied using the initial conditions. The flow rate was 1 mL min⁻¹, the injection volume 50 μL, the column temperature 28°C, and the detection wavelengths 250 nm for PB, 256 nm for EP, 272 nm for TH, and 285 nm for CD.

**Validation**

For validation purposes three bulk ‘model suppositories’ (Msup1–3) and a bulk ‘blank suppository’ were prepared. The active substances were accurately weighed and homogenized in a mortar with the contents of the appropriate number of Fenistil 24 retard capsules. The mixtures were finally suspended in the appropriate amount of gently melted hard fat. The compositions are listed in *Table I*.

*Table I. Composition of the model suppositories used for validation*

<table>
<thead>
<tr>
<th>Component name</th>
<th>Msup1</th>
<th>Msup2</th>
<th>Msup3</th>
<th>Blank suppository</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theophylline (mg)</td>
<td>100.0</td>
<td>300.0</td>
<td>300.0</td>
<td>0</td>
</tr>
<tr>
<td>Phenobarbital Na (mg)</td>
<td>25.0</td>
<td>75.0</td>
<td>75.0</td>
<td>0</td>
</tr>
<tr>
<td>Codeine HCl (mg)</td>
<td>100.0</td>
<td>300.0</td>
<td>300.0</td>
<td>0</td>
</tr>
<tr>
<td>Ephedrine HCl (mg)</td>
<td>100.0</td>
<td>300.0</td>
<td>300.0</td>
<td>0</td>
</tr>
<tr>
<td>Number of Fenistil capsules</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Hard fat (g)</td>
<td>20.0</td>
<td>30.0</td>
<td>20.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Number of dosage units</td>
<td>20</td>
<td>30</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Amount of active substances/suppositorya (%)</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>0</td>
</tr>
</tbody>
</table>

aRelative to the prescribed composition

Selectivity was tested by demonstrating freedom from interference from the matrix. Blank suppository (510 mg; equivalent to the sample size) was extracted and analyzed. Carry-over potential was investigated by injec-
tion of mobile phase A after a sample run. The working solutions for linearity testing were injected in duplicate. The mean response from the two injections for each level was plotted as a function of concentration. Correlation coefficients and distribution of residuals were determined. Repeatability (system precision) was assessed by injecting the reference solution five times and calculating the relative standard deviation (RSD) for each analyte. To determine intra-day and inter-day precision five independent samples of Msup2 were accurately weighed, extracted, and analyzed. The whole procedure was repeated with a different model (prepared by a different person but with the same composition) on a different day. RSD was calculated for both analyses. Accuracy was assessed by repeating this procedure on three samples each of Msup1 and Msup3 and calculating the recovery of all the active substances. Results for Msup2 were calculated from the combined method precision data. To assess stability, the reference solution was injected at the beginning and end of an overnight sequence of analyses. In addition, reference solution stored in a refrigerator (2–8°C) was analyzed 24 and 48 h after preparation.

Results and Discussion

Method and Extraction Development

The method was developed using the standard solutions of the active substances and Msup2. Sample preparation and the chromatographic conditions were designed taking into account the $pK_a$ values (Fig. 1) and solubility of the four compounds. Method development was based on the test for related substances described in the ‘Codeine hydrochloride dihydrate’ monograph of the 6th edition of the European Pharmacopoeia (Ph. Eur. 6.) [20], which prescribes use of a 250 mm × 4.6 mm C8 silica column (a C18 stationary phase was used instead) and a mobile phase consisting of 25% ($v/v$) ACN, 2% cc. CH3COOH, 73% water, and 1.08 g L$^{-1}$ NaOS as ion-pairing agent. For each analyte, the UV-DAD detection wavelength selected corresponded to an absorption maximum in the respective UV spectrum.

For each compound, individual standard solutions were prepared with the mobile phase prescribed in Ph. Eur. 6. to identify the corresponding peaks. Results indicated that this mobile phase composition led to co-elution of PB and EP, with CD eluting before these two compounds, and, furthermore, to a very low retention time for TH (retention factor, $k$, close to zero).
Reduction of the ACN content in the mobile phase resulted in a change in the order of elution of PB, CD, and EP and thus the problem of co-elution of PB and EP was eliminated. Even with further reduction of the ACN content to 15%, however, retention of TH was still unacceptably low. It was therefore suggested that gradient elution should be used. Use of mobile phase containing 10% ACN resulted in an acceptable retention for TH ($k \approx 1$ relative to the solvent peak), and after the elution of TH the ACN content of the mobile phase was increased to 20% by mixing ACN with the initial mobile phase consisting of 10% ACN, 2% CC. CH$_3$COOH, 88% water, and 1.08 g L$^{-1}$ NaOS. This stepwise gradient profile enabled separation of CD, PB, and EP in an isocratic step. This was deemed beneficial because isocratic elution generally results in better reproducibility and more precise quantification than a linear gradient [21–23].

The chromatograms obtained showed that the resulting decrease of the concentration of the IP agent NaOS in the mobile phase led to an irreproducible drift of the baseline and thus ACN was replaced by mobile phase component B. A typical chromatogram obtained under optimized conditions is presented in Fig. 2a. Performance data are listed in Table II.

**Fig. 2.** Chromatograms obtained, under optimized conditions, at 256 nm: (a) reference solution; (b) sample solution; (c) solution of the ‘blank suppository’
Table II. Performance data for the chromatogram depicted in Fig. 2a

<table>
<thead>
<tr>
<th></th>
<th>Theophylline</th>
<th>Phenobarbital</th>
<th>Codeine</th>
<th>Ephedrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention factor&lt;sup&gt;a&lt;/sup&gt; ($k'$)</td>
<td>0.878</td>
<td>6.915</td>
<td>8.036</td>
<td>10.083</td>
</tr>
<tr>
<td>Peak asymmetry&lt;sup&gt;b&lt;/sup&gt; ($A_S$)</td>
<td>1.179</td>
<td>1.100</td>
<td>1.108</td>
<td>1.285</td>
</tr>
<tr>
<td>Selectivity&lt;sup&gt;a, c&lt;/sup&gt; ($\alpha$)</td>
<td>–</td>
<td>2.592</td>
<td>1.162</td>
<td>1.255</td>
</tr>
<tr>
<td>Resolution&lt;sup&gt;a, c&lt;/sup&gt; ($R_S$)</td>
<td>–</td>
<td>19.460</td>
<td>4.455</td>
<td>5.921</td>
</tr>
</tbody>
</table>

<sup>a</sup>Apparent values for PB, CD, and EP, because of the gradient step
<sup>b</sup>Calculated as USP tailing factor at the wavelength used for the assay
<sup>c</sup>From the preceding peak

Liquid–liquid extraction was used for sample preparation. Because of the high lipophilicity of the hard fat CH₂Cl₂ was chosen as solvent. After addition of approximately 2.5 mL CH₂Cl₂ to an amount equivalent to half of the average weight of one dosage unit, complete dissolution of the hard fat was observed. The total amount of insoluble matter remaining (probably excipients from the Fenistil capsule) could be transferred to the separation funnel by addition of another 2.5 mL CH₂Cl₂.

The different acidic/basic character of the four analytes suggested quantitative extraction of the substances from the pharmaceutical formulation in one step might be problematic. Indeed, extraction with 3 × 5 mL acetate buffer (pH 5.0) resulted in acceptable recovery of TH (96.5%), CD (96.2%), and EP (99.5%), but for PB this method of sample preparation was ineffective, resulting in recovery of only 45.9%. To improve the solubility of PB in the aqueous phase, after extraction with acetate buffer the organic layer was extracted again with 3 × 5 mL 0.1 M NaOH. The increase in pH from 5.0 to approximately 13 resulted in complete ionization of PB thus increasing its solubility in water and resulting in recovery greater than 90%.

Because of the small amounts of the active substances, especially PB (one suppository contained only 2.5 mg), the automatic extraction process was thoroughly studied. Optimum mixing of the two phases occurred at 150 rpm. At a higher rate of rotation the two phases were swirled rather than mixed, which reduced the efficiency of extraction. An extraction time of 5 min was found to be sufficient to achieve acceptable recovery of TH, EP, and CD with pH 5.0 acetate buffer. Recovery of PB could be improved by increasing the extraction time with 0.1 M NaOH to 10 min.
Chromatography, and Results from Validation

On injection of the extract of the blank no peaks interfering with those of any of the analytes were detected (Fig. 2c). Injection of the mobile phase after a sample run resulted in no signs of carry-over. Regression equations for the calibration plots, and correlation coefficients, are presented in Table III. Residuals were randomly distributed.

Table III. Results from validation of the method

<table>
<thead>
<tr>
<th></th>
<th>Theophylline sodium</th>
<th>Phenobarbital hydrochloride</th>
<th>Codeine hydrochloride</th>
<th>Ephedrine hydrochloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity ((n = 6))</td>
<td>(y = 165.4x + 14.1; R^2 = 1)</td>
<td>(y = 10.37x - 1.166; R^2 = 0.9999)</td>
<td>(y = 12.36x - 6.582; R^2 = 1)</td>
<td>(y = 2.348x - 2.285; R^2 = 0.9999)</td>
</tr>
<tr>
<td>(S/N) for the lowest level</td>
<td>&gt;4000</td>
<td>20.9</td>
<td>48.7</td>
<td>12.5</td>
</tr>
<tr>
<td>Range ((\text{mg mL}^{-1}))</td>
<td>0.01-0.08</td>
<td>0.0025-0.02</td>
<td>0.01-0.08</td>
<td>0.01-0.08</td>
</tr>
<tr>
<td>System precision ((n = 5; RSD%))</td>
<td>0.11</td>
<td>1.37</td>
<td>0.50</td>
<td>0.70</td>
</tr>
<tr>
<td>Method precision ((n = 5))</td>
<td>Day 1 mean</td>
<td>98.5</td>
<td>90.2</td>
<td>97.1</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>2.03</td>
<td>1.20</td>
<td>1.99</td>
<td>3.24</td>
</tr>
<tr>
<td>Day 2 mean</td>
<td>99.1</td>
<td>93.3</td>
<td>97.9</td>
<td>97.4</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>0.42</td>
<td>1.19</td>
<td>0.52</td>
<td>1.57</td>
</tr>
<tr>
<td>Accuracy (as recovery, %)</td>
<td>At 50% API content ((n = 3))</td>
<td>99.8 ((\text{RSD 0.25%}))</td>
<td>91.0 ((\text{RSD 1.00%}))</td>
<td>98.5 ((\text{RSD 0.54%}))</td>
</tr>
<tr>
<td>At 100% API cont. ((n = 10))</td>
<td>98.8 ((\text{RSD 1.41%}))</td>
<td>91.7 ((\text{RSD 2.10%}))</td>
<td>97.5 ((\text{RSD 1.44%}))</td>
<td>96.9 ((\text{RSD 2.46%}))</td>
</tr>
<tr>
<td>At 150% API cont. ((n = 3))</td>
<td>99.3 ((\text{RSD 0.19%}))</td>
<td>96.4 ((\text{RSD 0.86%}))</td>
<td>98.6 ((\text{RSD 0.50%}))</td>
<td>99.6 ((\text{RSD 0.37%}))</td>
</tr>
</tbody>
</table>

\(^{a}\)Mean recovery and RSD calculated from the combined results from study of method precision on day 1 and day 2.
In assay methods, determination of the limits of detection (LOD) and quantification (LOQ) is not required by the corresponding ICH guideline [19]. Because of the small amounts of the active pharmaceutical ingredients (APIs) in the suppository and the low concentrations of sample solutions, however, signal-to-noise ratios ($S/N$) were calculated for the least concentrated solutions used for linearity. As shown in Table III, $S/N$ was greater than 10 for all the analytes which, by convention, represents the LOQ. Therefore, the 0.01–0.08 mg mL$^{-1}$ working range for TH, CD hydrochloride, and EP hydrochloride and 0.0025–0.02 mg mL$^{-1}$ range for PB sodium was confirmed. Results from determination of system precision (repeatability) and intra-day and inter-day precision are shown in Table III. Mean and RSD values obtained in the two tests are comparable for all the four APIs. The results presented in Table III show recovery was acceptable (>95%) for TH, CD hydrochloride, and EP hydrochloride at all three levels. For PB sodium recovery was below 95% but still greater than 90% which was deemed acceptable, because PB sodium was the API at the lowest level in the suppository (2.5 mg) and thus the test solution concentrations were much lower than for a usual assay. Furthermore, PB was the only component which could not be extracted with the acetate buffer in one step which might contribute to the reduced method accuracy. No signs of degradation were observed for any of the analytes in the reference solution. As a result, it was concluded that the reference solution is stable at room temperature for the duration of a complete sequence of analyses (12 h) and can be stored at 2–8°C for at least 48 h.

**Sample Testing**

A suppository of quality concern submitted by a customer was analysed by use of the validated method. A 13-month-old baby was reported to have shown symptoms of intoxication after administration of the sample. Results from the analysis are presented in Table IV. A typical chromatogram is presented in Fig. 2b. Analysis of the sample and the appropriate system-suitability tests were performed within one working day.

According to the national guideline applied by the Hungarian authority for quality control of magistral preparations, non-compliance is indicted if results are outside the range 85–115% of the nominal content. It was found that none of the active substances complied with this requirement. It was also found that the amount of CD hydrochloride (12.6 mg in one dosage unit) slightly exceeded the maximum single dose of 12 mg stipulated for a one-year-old child by the 7th edition of the Hungarian Pharmacopoeia [24]. Hence, it was suggested that the adverse event might be related to an overdose of CD.
Table IV. Results from analysis of the tested suppository of quality concern (percentage of nominal content)

<table>
<thead>
<tr>
<th></th>
<th>Theophylline</th>
<th>Phenobarbital sodium</th>
<th>Codeine hydrochloride</th>
<th>Ephedrine hydrochloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Result from assay (%; mean; n = 6)</td>
<td>122.6</td>
<td>115.7</td>
<td>126.5</td>
<td>131.4</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>1.20</td>
<td>2.76</td>
<td>0.90</td>
<td>1.50</td>
</tr>
</tbody>
</table>

Conclusions

Extemporaneous pharmaceutical preparations may contain numerous APIs which might differ with regard to amount present, solubility, acidic/basic character, etc. Analysis of such products may be demanding if there are many components present at low levels compared with the excipients. Analysis of the active substances in suppositories can be achieved by HPLC after extraction from an organic phase with one or more different aqueous solvents. The method described in this paper was successfully used to confirm a suspected quality problem of a magistral suppository. It may also serve as a basis for elaboration of other methods for testing suppositories of similar composition.

References


Accepted by DA